Muscle derived stem cells in the treatment of anal sphincter injury in a rat model – An interventional study

A dissertation submitted in partial fulfillment
of the requirement towards
The Tamil Nadu Dr. M. G. R. Medical University
for the M.S. Branch-I (General Surgery)
Examination to be held in May 2018

DECLARATION CERTIFICATE

This is to declare that the dissertation titled “Muscle derived stem cells in the treatment of anal sphincter injury in a rat model – An interventional study” in the department of general surgery is my own work, done under the guidance of Dr. Sukria Nayak, Professor and Head, Department of General Surgery Unit-IV, Christian Medical College, Vellore and is being submitted in partial fulfillment of the rules and regulations for the M.S Branch I – General Surgery degree examination of The Tamil Nadu Dr. M.G.R Medical university, Chennai, to be held in May 2018.

Dr. Sasank Kalipatnapu
MS Post Graduate Registrar
Department of General Surgery
Christian Medical College, Vellore.

BONAFIDE CERTIFICATE

This is to certify that “Muscle derived stem cells in the treatment of anal sphincter injury in a rat model – An interventional study” is a bonafide work of Dr. Sasank Kalipatnapu, in partial fulfillment of the requirements for the M.S. General Surgery examination (Branch I) of The Tamil Nadu DR M.G.R. Medical University to be held in May 2018.

Dr. Sukria Nayak	Dr.Sukria Nayak	Dr.Anna B.Pulimood
Guide	Head of the Department	Principal
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December 28, 2015

Dr. Sasank K
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Sub: **Fluid Research Grant NEW PROPOSAL:**
Muscle derived stem cells in the treatment of anal sphincter injury in a rat model – an interventional study
Dr. Sasank K, (Employment Number: 29473), Department of Surgery IV, Dr. Sukriya Nayak, Employment Number: 30005, Surgery IV, Dr. Suchita chase (Emp. No. 13572), General Surgery, Dr. Vrisha Madhuri (Emp. No. 08689), Paediatric Orthopaedics, Dr. Vasanth Mark Samuel (Emp.No. 33291), Surgery, Dr. Suresh Devasahayam (Emp. No. 15028), Bioengineering, Mr. Prasanna Samuel (Emp. No. 31654), Biostatistics, Dr. Geeta Chacko (Emp. No. 11713), Pathology, Dr. Bimal Patel (Emp. No. 33508), Pathology

Ref: IRB Min No: 9653 [INTERVEN] dated 23.09.2015

Dear Dr. Sasank K ,
The Institutional Review Board (Silver, Research and Ethics Committee) of the Christian Medical College, Vellore, reviewed and discussed your project titled "Muscle derived stem cells in the treatment of anal sphincter injury in a rat model – an interventional study" on September 23rd 2015.

The Committee reviewed the following documents:

1. IRB Application format
2. Data Collection Proforma
3. Cvs of Drs. Sasank K, Sukriya Nayak, Suchita chase, Vrisha Madhuri, Bimal Patel, Geeta Chacko, Vasanth Mark Samuel
4. No. of documents 1 - 4

The following Institutional Review Board (Silver, Research & Ethics Committee) members were present at the meeting held on September 23rd 2015 in the CREST/SACN Conference Room,Christian Medical College, Bagayam, Vellore 632002.

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Dr. Prasanna Samuel	MSc, PhD	Lecturer, Biostatistics, CMC, Vellore	Internal, Statistician
Dr. B. Poonkuzhali	MSC, PhD	Professor, Haematology, CMC, Vellore	Internal, Basic Medical Scientist
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Dr. Nihal Thomas	MD, MNAMS, DNB(Endo), FRACP (Endo) FRCP(Edin) FRCP (Glasg)	Professor & Head, Endocrinology, Additional Vice Principal (Research), Deputy Chairperson (Research Committee), Member Secretary (Ethics Committee), IRB, CMC, Vellore	Internal, Clinician

We approve the project to be conducted as presented.

The Institutional Ethics Committee expects to be informed about the progress of the project, any **adverse events** occurring in the course of the project, any **amendments in the protocol and the patient information / informed consent**. On completion of the study you are expected to submit a copy of the **final report**. Respective forms can be downloaded from the following link:

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http://172.16.11.136/Research/IRB_Polices.html in the CMC Intranet and in the CMC website link address: http://www.cmch-vellore.edu/static/research/Index.html.

Kindly provide the total number of patients enrolled in your study and the total number of withdrawals for the study entitled: "Muscle derived stem cells in the treatment of anal sphincter injury in a rat model – an interventional study" on a monthly basis. Please send copies of this to the Research Office (research@cmcvellore.ac.in)

Fluid Grant Allocation:

A sum of 1,00,000/- INR (Rupees One Lakh only) will be granted for 2 year and out of which a maximum of Rs.5000/- can be spent for stationery, printing, Xeroxing and computer charges(If computers used are within the institution)

Yours sincerely

Dr. Nihal Thomas
Secretary,(Ethics Committee).
Institutional Review Board
Christian Medical College, Vellore.

Dr. NIHAL THOMAS
MD, MNAMS, DNB(Endo), FRACP(Endo), FRCP(Edin), FRCP(Glasg)
SECRETARY - (ETHICS COMMITTEE)
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Cc: Dr. Sukriya Nayak, Dept. of Surgery IV, CMC.

IRB Min No: 9653 [INTERVEN] dated 23.09.2015

INSTITUTIONAL ANIMAL ETHICS COMMITTEE
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Dr. Alfred Job Daniel
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email: princi@cmcvellore.ac.in

Dr. Vinay Timothy Oommen
Secretary
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8th February 2016

To
Dr. Sasank K
PG Registrar,
Department of Surgery III
CMC

Dear Dr. Sasank K

Your research proposal titled **"Muscle derived stem cells in the treatment of anal sphincter injury in a rat model - an interventional study"** has been reviewed by the Institutional Animal Ethics Committee (IAEC) on 1st February 2016.

After discussion, **19 SD rats for Year I** have been approved for the study

Location of experiments: **Stem Cell Facility**

The IAEC approval number for the study is **2/2016**

You are required to maintain all records as per form D, ensure humane treatment of animals and submit a **final report** to the IAEC. If an extension or the use of new animals after the sanctioned period is required, a **progress report** must be submitted to the IAEC with a request for new animals.

With best wishes,
Yours sincerely,

Dr. Alfred Job Daniel,
Principal & Chairperson
Institutional Animal Ethics Committee

Cc:
Dr. Vinay Timothy Oommen
Secretary, IAEC

URKUND

Urkund Analysis Result

Analysed Document: Urkund submission.pdf (D31347276)
Submitted: 10/15/2017 11:57:00 PM
Submitted By: ksasank@gmail.com
Significance: 1 %

Sources included in the report:

BMW thesis final edited.docx (D31180568)

Instances where selected sources appear:

1

ACKNOWLEDGEMENTS

"An ambitious thesis plan", I was told by several people when I started off on this study. As the days went by, I grew in to caution and wisdom in that statement.

Now, as I look back, this learning experience has come about by contributions of many – seen and unseen, expected and unexpected, planned and unplanned. My gratitude –

To God – for making the impossible possible

To Dr.Sukriya Nayak, Dr.Suchita Chase for their unwavering faith in my ability to finish this study; for giving me the freedom to do so at my own pace; for constantly encouraging and entertaining all the spontaneous ideas I would come up with.

To Dr.Vrisha Madhuri – This study would not have happened if it hadn't been for her guidance and support both in terms of academic and laboratory.

To the "Lab 4" team from Centre for Stem cell Research, it has been an amazing journey with them, from lab sessions to dinners to animal surgery. They had always always been there when I needed them.

To Dr.Anand Bhaskar, for single-handedly rescuing my anal manometry for this study.

To Dr.Bimal Patel, for putting up with my continuous badgering and willing to work after hours to finish this project

To the Animal lab facility team – Dr.Arun, Mrs.Pavithra, Mr.Ashok –and the histology team - Mrs.Esther – for their ever prompt answers and having been so accommodative of my requests always.

To the Institutional Review Board and Animal Ethics Committee for their permission to embark on this study.

Dedicated to –

Sowmya Ramesh for being the keystone in this study

My mother who always taught me to have faith – in God and myself.

ABSTRACT

Title of the Abstract: Muscle derived stem cells in the treatment of anal sphincter injury in a rat model – An interventional study

Department: General Surgery

Name of the Candidate: Dr. Sasank K

Degree and Subject: MS (General Surgery)

Name of the Guide: Dr. Sukria Nayak

Objectives:

1. To standardize a rat animal model of anal sphincter injury using functional and histological parameters
2. Quantification of the anal sphincter contractility at baseline, after injury and after injection of stem cells
3. Histological examination of the anal sphincter to look for structural regeneration of the sphincter muscle fibres

Methods:

A prospective cohort study was designed using two arms – control and test arm. The baseline manometry and anal sphincter contractility were measured for all rats, following which the rats underwent partial sphincter excision. The manometry was repeated in all the rats after the injury to demonstrate anal sphincter insufficiency. The stem cells were harvested from the hind limb muscle of the same animals in the test group under the same anaesthesia. Muscle derived stem cells were isolated from the muscle sample and then injected back into the anal sphincter after allowing adequate wound healing. The control rats received a placebo injection of phosphate buffered saline. All animals were followed up at a mean follow up of 5 weeks and underwent an anal manometry, following which they were sacrificed and their anal sphincter complex was subjected to histopathological examination.

Results:

A total of 11 animals were included in the study – 5 in the test arm and 6 in the control arm. All animals tolerated the procedure well. The hind limb muscle biopsy was a good source to isolate satellite cells. The anal manometry of both the control and test arm animals reached normal values by 1 month follow up. However, on histopathological examination, there was unorganised muscle in the area of defect in the animals injected with stem cells while there was predominantly fibrosis in the defect in animals injected with only phosphate-buffered saline.

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Introduction

"Civilisation rests on two things," said Hitzig; "the discovery that fermentation produces alcohol, and voluntary ability to inhibit defecation. And I put it to you, where would this splendidly civilized occasion be without both?"

Robertson Davies, The Rebel Angels (Cornish Trilogy #1)

Continence to faeces and flatus plays an important role in our everyday functioning in the society. It enables us to move around freely without the worry of soiling ourselves or the consequent embarrassment. The impact of incontinence on the personal, family and social life of a person is not to be doubted upon. The most common cause continues to be traumatic anal sphincter injuries following vaginal delivery. (1) Treatment options for large segment injuries or irregular injuries of anal sphincter remain limited in availability and efficacy. (2)

The idea of regenerative medicine has taunted mankind since time immemorial. As traditional methods of anal sphincter repair have had mixed results, the search for the best treatment continues and there are studies which propose stem cell based therapy as the holy grail of treatment of anal incontinence. (3-5)

Aims

To study the use of muscle derived stem cells in the treatment of anal sphincter injury in a rat model

Objectives

1. To standardize a rat animal model of anal sphincter injury using functional and histological parameters
2. Quantification of the anal sphincter contractility at baseline, after injury and after injection of stem cells
3. Histological examination of the anal sphincter to look for structural regeneration of the sphincter muscle fibres

Literature Review

Overview

1. Anatomy of the continence mechanism
2. Physiology of the continence mechanism
3. Faecal & Anal incontinence
4. Stem cells
5. Justification for the study

Anatomy of the continence mechanism

Anal Canal

The anal canal is a muscular tube measuring 3-5 cm extending from the anorectal junction till the intersphincteric groove (approximately 2cm from the dentate line).(6) The anorectal junction is located at the palpable upper border of the anal sphincter mechanism -the junction of the puborectalis and the anal sphincter. The anal margin is that portion of the skin from the intersphincteric groove extending outwards for a distance of 5 cm. (7)

The musculature of the anal canal can be visualised as two concentric layers.

1. The outer funnel shaped layer is formed by the levator ani in the upper half and the external anal sphincter in the lower half. Histologically, this layer is skeletal muscle and is supplied by somatic nerves.(7)

2. The inner cylindrical tube is formed by the internal anal sphincter. The internal sphincter is merely a thickened extension of the longitudinal layer of muscle of the rectum. This layer is histologically smooth muscle and is supplied by autonomic nerve fibres along the inferior rectal nerve. (7)

The anorectal junction is marked by an acute angulation produced by the forward pull of the puborectalis sling. Lateral relations of the anal canal are the ischiorectal fossae and, anteriorly, it is related to the urethra in men and the lower vagina in women.(6)

Rectum

The rectum extends from the sacral promontory to the anorectal ring – the junction of the levator ani and the anal sphincter complex. The taenia coli of the colon coalesce to form the longitudinal layer of the rectum and the appendices epiploicae disappear at the upper end of the rectum. The upper border of rectum extends for 10-15 cm from the anal verge. Surgically, the upper border of rectum is taken to be overlying the sacral promontory while anatomically, the rectum starts opposite the body of S3, or 14 to 16 cm from the anal verge. (6)

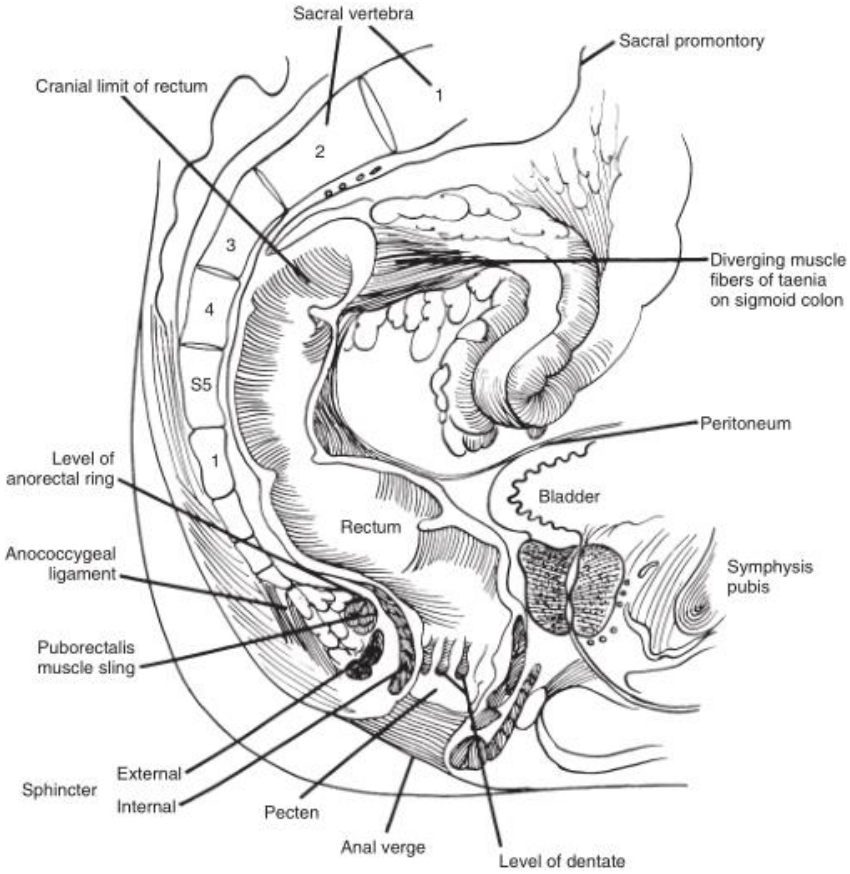


Figure 1. The course of the rectum through the pelvis.
(Reproduced from Shackelford's Surgery of the Alimentary tract, 7th Edition, Elsevier Saunders, PA)

Pelvic floor musculature

The pelvic floor helps to support the pelvic and abdominal organs and is formed by two symmetric sets of muscles that interlink in the midline as a raphe. It helps to support the abdominal and pelvic organs inferiorly. (6) The anatomy of the pelvic floor is as shown in Figure 2.

The bulbous central portion of the perineum is formed by the *perineal body*. The bulbospongiosus, external sphincter, superficial and deep transverse perineal muscles insert into the perineal body and constitute a crucial support to the perineum and vagina. Anterior sphincter repairs for faecal incontinence invariably require reconstruction of the perineal body. (6)

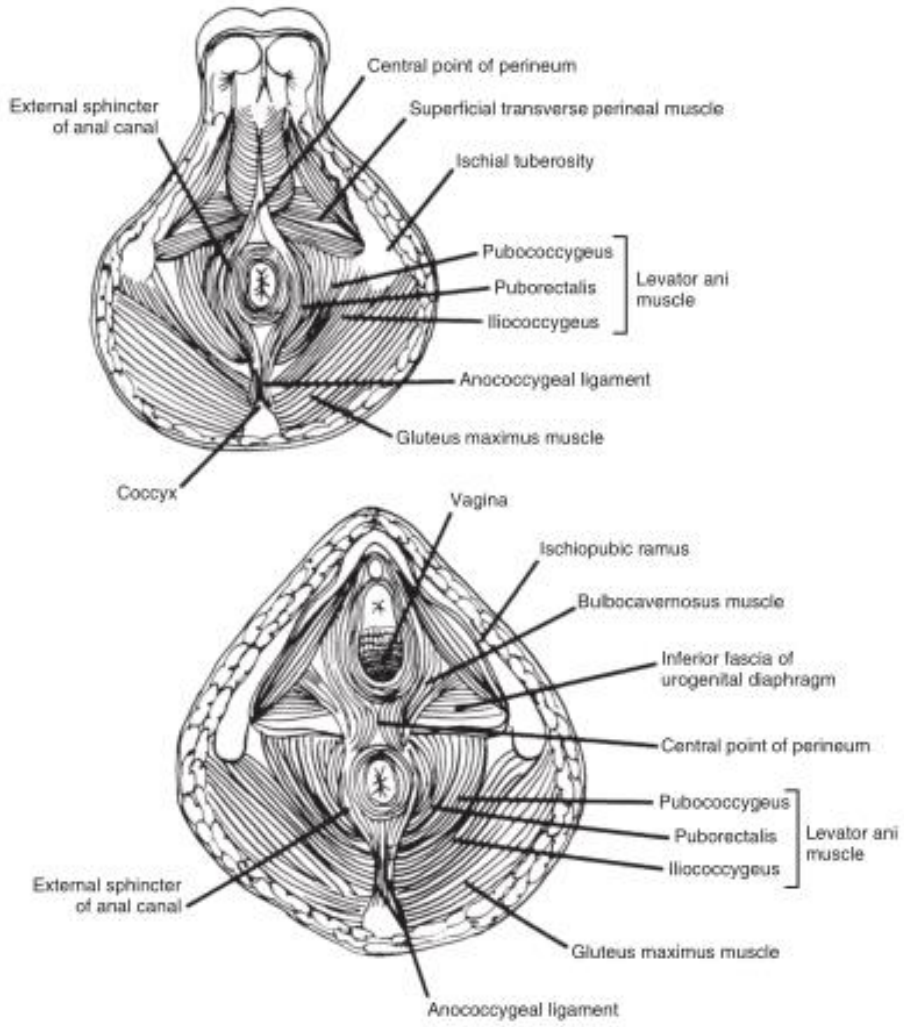


Figure 2. The levator ani muscles in men and women.
(Reproduced from Shackelford's Surgery of the Alimentary tract, 7th Edition, Elsevier Saunders, PA)

Physiology of continence

Faecal continence is defined as the ability to defer defecation till a socially acceptable time and place can be found. The physiological mechanism of continence is complex and is controlled by a myriad of anatomical, physiological and neurological factors. (7)

Anal canal pressure and anal sphincters

The internal and external sphincters, as mentioned above, form two concentric tubes around the anal canal.

a) The internal sphincter is tonically contracted at rest and contributes to 85% of the resting sphincter tone.
b) The external sphincter, though a striated muscle has a basal tone, which it maintains during the day and to a lesser extent, during sleep.

Squeeze pressures are more than twice the resting pressure during maximum effort. This is generated by the voluntary contraction of the puborectalis and the external anal sphincter.

The sphincter pressures at rest varies along the longitudinal length of the anal canal as shown in the graph below. (7)

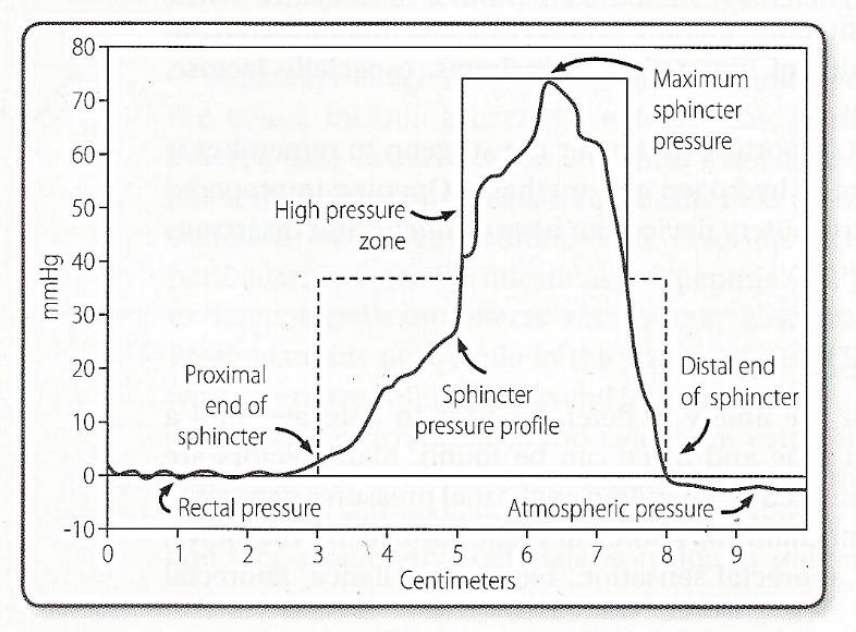


Figure 3.Characteristic of a typical longitudinal pressure profile of the resting sphincter. (Reproduced from Handbook of Colorectal Surgery, 3rd Edition, JP Medical Publishers, New Delhi, India)

Anorectal angle

The angle formed between the longitudinal axis of the anal canal and the rectum is defined as the anorectal angle. It is maintained by the tonic contraction of the puborectalis muscle. At rest in left lateral position, the mean ± SD angle was 102 ±18 degrees. This angle sharpened to 87 ± 23 degrees on Valsalva maneuver which stressed the continence mechanism. (6)

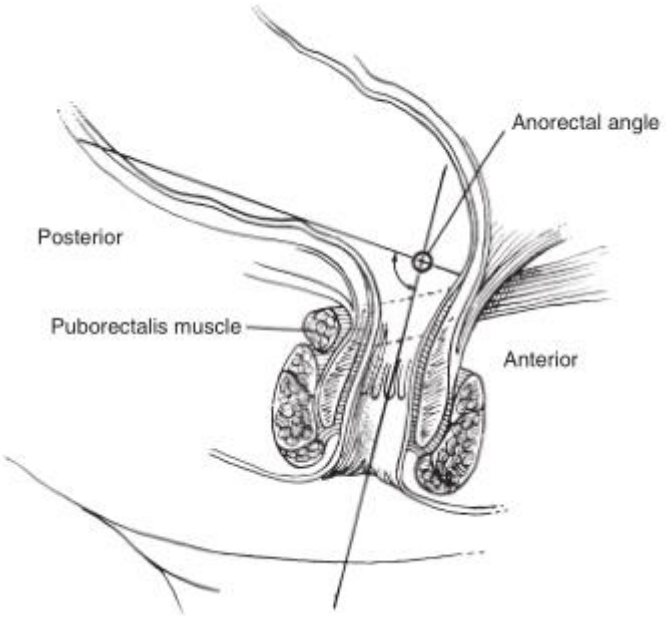


Figure 4. Anorectal angle –It is measured between a line drawn through the centre of the anal canal and along the posterior wall of the rectum. (Reproduced from Shackelford's Surgery of the Alimentary tract, 7th Edition, Elsevier Saunders, PA)

It is an important structural component to the continence mechanism and aids in preventing leakage of solid matter, even if the sphincter is inadequate. Squatting straightens the anorectal angle to >110 degrees and this is augmented by straining, which renders the puborectalis and external anal sphincter electrically silent. (6)

Rectal Compliance, Tone and Capacity

In continent patients, there is a pressure gradient between the high resting pressures in the anal canal and low pressures in the rectum. A compliant rectum is crucial to the maintenance of low rectal pressure and the pressure gradient. As the volume of contents increases, the compliance of the rectum or *receptive relaxation* aids in the

distension of the rectum while keeping the intraluminal pressures low. The pressure increases only after the volume increases beyond 300 ml. The mean rectal compliance in a normal individual ranges from 4 to 14 mL/cm H_2O. (6)

Defaecation reflex

Normal defaecation occurs using the defaecation reflex which is given in Figure 5.

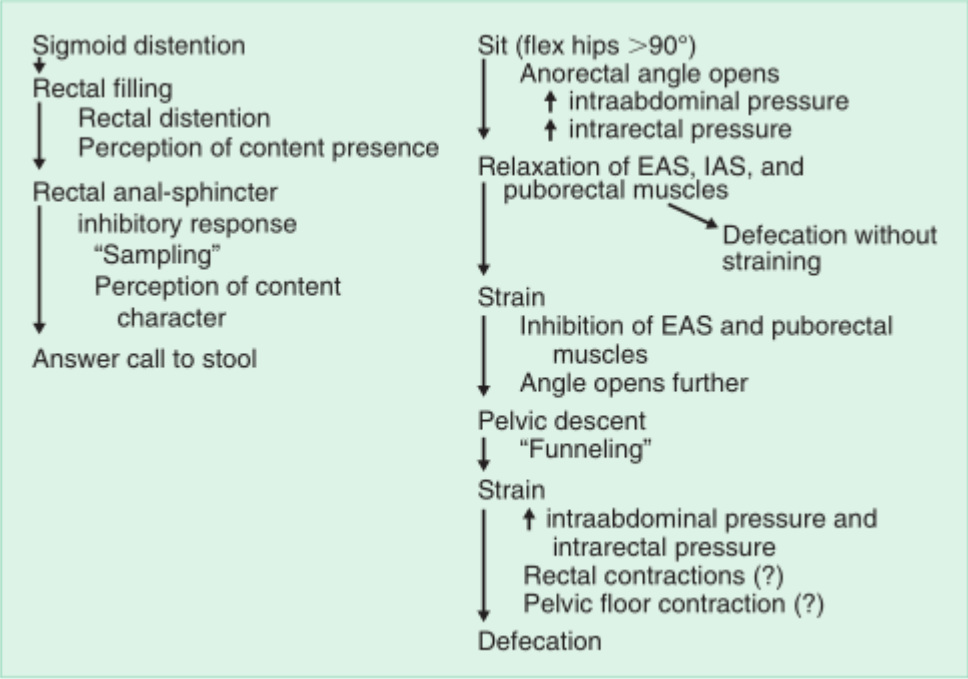


Figure 5. Sequence of Defecation
(Reproduced from Shackelford's Surgery of the Alimentary tract, 7th Edition, Elsevier Saunders, PA)

Faecal incontinence

Faecal Incontinence (FI) is defined as "*the uncontrolled passage of faeces or gas over at least 1 month's duration, in an individual of at least 4 years of age, who had previously achieved control". (8)* The Rome criteria defines it as "continuous or recurrent uncontrolled passage of faecal material (>10 mL) for at least 1 month in an individual."(9) A general definition would be the inability to control the release of rectal contents until a socially acceptable time and place.(7) It has also been defined as unintentional loss of solid/liquid stool and anal incontinence includes leakage of gas and/or FI.(10)

Epidemiology

Measurement of the exact prevalence of faecal incontinence in the general population has been difficult as it is very often not reported by the patient. (10) Its impact on the social and emotional lives of people, especially the elderly population, leads to a poor quality of life. (10, 11)

Lack of effective and appropriate treatment modalities coupled with chronic disabling symptoms has led to recognition of faecal incontinence as an economic and public health issue. (12, 13) The reported prevalence ranges from 7-15 % among men and women each in the general population. (10) These rates however differ based on the target population measured, the method used to estimate prevalence, the questions used and the definition of incontinence used.(8, 10)

In a population study of women in the reproductive age group, the prevalence was 4.4%, while in a geriatric population in a nursing home, it was as high as 50%.(14, 15) After adjusting for age, comorbid illnesses and BMI, independent risk factors for faecal incontinence among women were found to be chronic diarrhoea, depression, white race and urinary incontinence. Among men, only urinary incontinence was an independent risk factor.(10) Other risk factors included physical factors such as immobilisation, use of physical restraints; chronic medical conditions such as diabetes mellitus, Parkinson's disease, stroke, urinary incontinence; surgical procedures such as lateral anal sphincterotomy, fistulotomy or ileal pouch reconstruction; obstetric factors such as prior vaginal delivery. Black or American Indian/Alaska Native race, income ≥ $40,000 per year and being married were associated with a decreased odds of FI in women. Routine screening among patients for faecal incontinence by using leading questions may be helpful in identifying “silent sufferers” of faecal incontinence. (15, 16)

An internet based survey involving approximately 6000 women >45 years of age in the United States revealed that upto 20% of women had experienced faecal incontinence atleast once in a year and 97% of them were concerned about it. Of the total sample, 938 women fit the criteria for diagnosis of faecal incontinence and among them, two thirds (71%) preferred the term 'accidental bowel leakage' over faecal or bowel incontinence. (16)

Perception of people towards faecal incontinence may also play an important role in health seeking behavior of patients afflicted with faecal incontinence. Many may

dismiss their symptoms as a part of normal aging, others suffer from pessimism towards physicians and disbelief in the availability of treatment modalities for faecal incontinence. (10)

Etiology

Faecal incontinence can be caused by multiple mechanisms arising out of a multitude of conditions. These are summarized in Table 1.(17, 18)

Insufficiency of the external anal sphincter, either neurological or myogenic, is the most common pathogenetic mechanism of faecal incontinence. While direct mechanical damage leads to myogenic dysfunction, neurological etiology involves either spinal or peripheral nerves disruption—in most cases the pudendal nerve.(19) The most common cause continues to remain obstetric anal sphincter injuries. (20)

Scoring Systems

Quality of life scoring systems aid in monitoring the response of the patient to therapy. Several scoring systems have been designed. A simple system was proposed by Parks - A, being normal; B, incontinent to flatus; C, incontinent to liquid stool; and D, incontinent to solid stool. In 1993, Jorge and Wexner proposed a system which has now become widely known as the Cleveland clinic incontinence score (Figure 6). (18)

Table 1. Pathogenesis and etiology of Faecal incontinence

Pathogenetic Mechanism	Etiology
Anal sphincter weakness	
• **Injury**	Obstetric Trauma, Post surgical procedures (e.g. internal sphincterotomy)
• **Non-traumatic**	Internal sphincter thinning of unknown etiology, scleroderma
• **Neuropathy**	Diabetes mellitus
Anatomical disturbances of pelvic floor	Rectal prolapse, descending perineum syndrome
Anorectal inflammation	
• **Increased intestinal propulsion**	Crohn's disease, Ulcerative colitis, anorectal infection
• **Reduced Rectal Capacity**	Anterior resection, Radiation proctitis
Central Nervous system disease	Stroke, Brain tumours, Spinal cord lesions, Multi-system atrophy (Shy Drager's syndrome), multiple sclerosis, Dementia
Bowel disturbances	Diarrhoea and constipation with/without faecal impaction
Congenital	Congenital ano-rectal malformation

TABLE 16-1 The Jorge–Wexner Incontinence Scoring System

	FREQUENCY				
Type of Incontinence	**Never**	**Rarely**	**Sometimes**	**Usually**	**Always**
Solid	0	1	2	3	4
Liquid	0	1	2	3	4
Gas	0	1	2	3	4
Wears pad	0	1	2	3	4
Lifestyle alteration	0	1	2	3	4

0 = Perfect
20 = Complete incontinence
Never = 0 (never)
Rarely = <1/month
Sometimes = <1/week, >1/month
Usually = <1/day, >1/week
Always = >1/day

Figure 6. Cleveland Clinic incontinence Score.
(Reproduced from Corman's Colon and Rectal Surgery, 6th Edition)

Management

The choice of investigations depends on the likely cause of the incontinence – mechanical or functional. (18) An endoanal ultrasound and anorectal manometry form the basis for the treatment of most patients with incontinence. Oher physiological studies including defaecography, cineradiography and estimation of intestinal transit are indicated if the predominant symptom is difficulty in evacuation. If the primary reason is likely neurological, it would require investigation for the same. (18)

All patients would require imaging of the sphincter complex to delineate the extent of injury – endoanal ultrasonography or MRI of the anal sphincter complex. Further management strategy would depend on the following –

1. Severity of incontinence
2. Structural integrity of the anal ring – inspection & anal ultrasound
3. Area and extent of weakness of the sphincter – palpation & ultrasound
4. Rectal sensations – clinical, electrical sensitivity, balloon volumetry

Current available treatment modalities for faecal incontinence includes behavioural, medical and surgical methods. Emerging therapies include tibial/pudendal nerve stimulation, anal plugs, vaginal balloon devices and mesh sling support for anorectal angle. (21)

The various methods are listed in Figure 7.

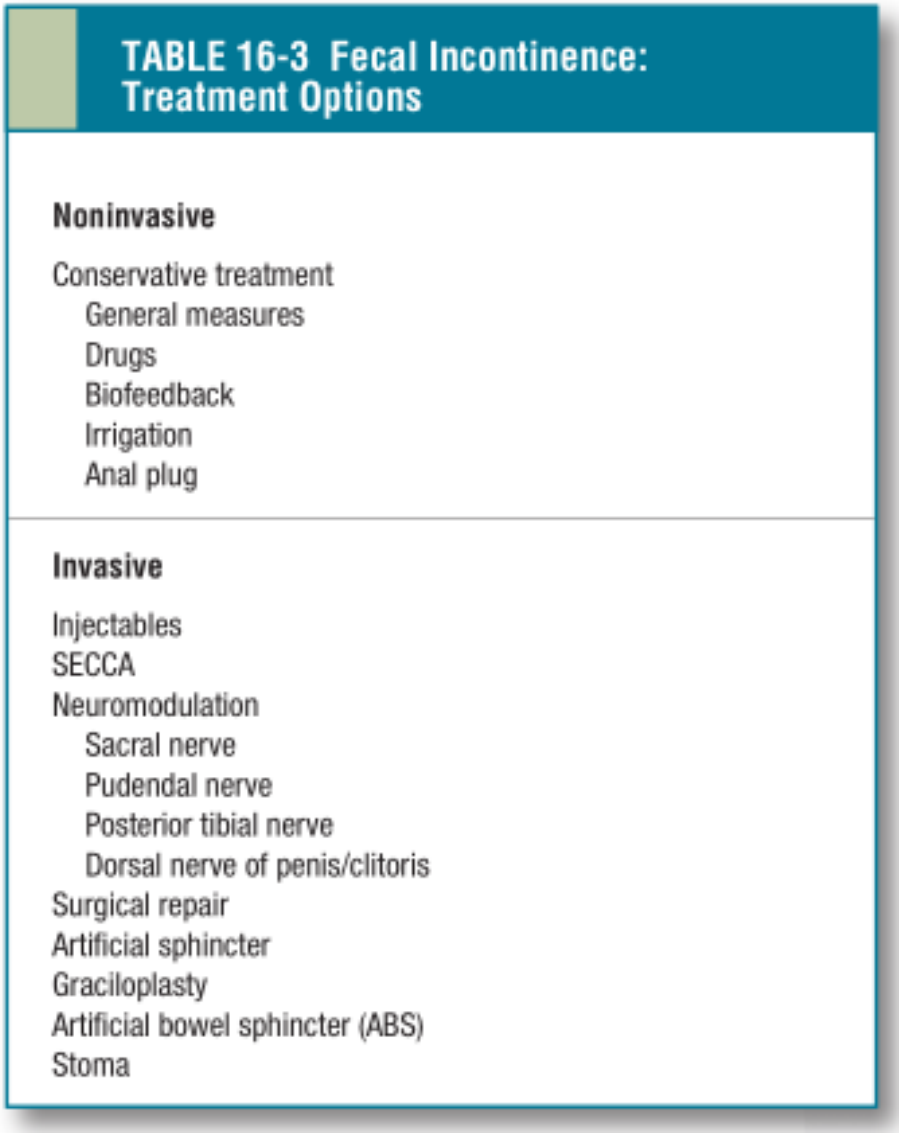


TABLE 16-3 Fecal Incontinence: Treatment Options

Noninvasive

Conservative treatment
- General measures
- Drugs
- Biofeedback
- Irrigation
- Anal plug

Invasive

Injectables
SECCA
Neuromodulation
- Sacral nerve
- Pudendal nerve
- Posterior tibial nerve
- Dorsal nerve of penis/clitoris

Surgical repair
Artificial sphincter
Graciloplasty
Artificial bowel sphincter (ABS)
Stoma

Figure 7. Treatment options for faecal incontinence.
(Reproduced from Corman's Colon and Rectal Surgery, 6th Edition)

Algorithm for management

In general, conservative management should be attempted unless invasive treatment is definitely indicated or inevitable. However, this depends on the severity of the symptoms and if there would be adequate improvement with non-invasive techniques. If so, then conservative management should be attempted as the initial treatment of choice.

A general algorithm for the choice of treatment modality is given in Figure 8. A patient with a complete sphincter will not respond to surgical techniques because there is no anatomical defect to repair. In such condition, non-invasive management remains the main modality of treatment. However, if they should fail, options include neuromodulation, anal canal injections or colonic irrigation.

A patient with a sphincter defect is a potential candidate for surgical repair if the defect is large. If the defect is small, neuromodulation could also be attempted. Failure of first sphincter repair should not be a deterrent from attempting a second repair, if the contractility of the sphincter is preserved.(18)

TABLE 16-4 Algorithm for the Management of Fecal Incontinence

Sphincter intact	Medical treatment Failure → SNS Failure → ? Artificial sphincter Failure → Colostomy
Sphincter defect	Repair if large, SNS if small Failure → Repeat repair or SNS Failure repeat repair → SNS Failure SNS → ? Artificial sphincter/colostomy

SNS, sacral nerve stimulation.

Figure 8.Algorithm for management.
(Reproduced from Corman's Colon and Rectal Surgery, 6th Edition)

Self management

Patients with small volume faecal soiling can manage by themselves by using absorbent pads. However, data pertaining to patient satisfaction is lacking. (21)

Conservative management

It includes measures such as education, promotion of healthy living, dietary advice and drug treatment.

General measures

They include education of the patient to the anatomy and function of pelvic floor as well as the mechanism of defecation. Weight reduction and daily exercise should be routinely recommended to everyone. Smoking may reduce intestinal transit time and worsen the symptoms. Hence, cessation of smoking can improve symptoms.

Drug treatment for incontinence includes anti-diarrhoeal agents as well as those used to treat constipation. Loperamide is generally considered the drug of choice. It is usually tolerated well with no irreversible side effects. (18)

Kegel's Exercises

Proposed in 1950, they have been found to be beneficial in both faecal and urinary incontinence. They help by increasing the muscle bulk and contractility of the external anal sphincter, puborectalis sling and levator ani muscles. (18)

Biofeedback

This is the preferred first line of management. It involves the combination of monitoring of anal pressure, which the patient can associate to his/her own attempts at contraction. In upto 70% of patients, it has shown improvement primarily in mechanical causes. However, it is limited by poor response following neurological damage. (18, 22-24)

Anal plug

Though designed initially for control of faeces from an end colostomy, it has hence been adapted for anal use. However, it causes significant amount of discomfort to the patient. (18)

Colonic Irrigation

It involves washing out of the colon using either antegrade or retrograde approach to decrease the faecal soiling due to incontinence. Several techniques and devices have been described for both the antegrade and retrograde approach. (18)

Invasive techniques

Injection of bulking agents around the anal canal

Started in 1993, it involves injecting inert substances (e.g. dextranomer microspheres, carbon coated zirconium beads, ceramic beads silicone elastomers, Teflon, silicone) around the anal canal to increase resting anal canal pressures. Early studies failed to demonstrate any significant symptomatic improvement. A recent large multicentric trial showed a sustainable significant improvement with dextranomer microsphere injection as compared to placebo injections. Out of a population of people with at least moderate severity of faecal incontinence, defined as per the Cleveland Clinic Florida incontinence score, 51% had significant improvement as compared to only 31% with placebo injections. (21)

It improves passive incontinence in the short term in >50% of patients, but improvement is usually temporary and may require repeated injections. It can be done as an out-patient procedure. (18)

Radiofrequency Energy Delivery for the Treatment of Faecal Incontinence (SECCA Procedure)

Radiofrequency energy results in tissue heating by vibration of water molecules. The Secca System (Mederi Therapeutics, Inc., Greenwich, CT) was targeted to induce fibrosis in the anal canal by RF induced energy. It has been mainly tested for patients with passive incontinence. However, small studies with limited data has precluded the acceptance of this therapy into mainstream practice. (18)

Neuromodulation / Electrical stimulation from anal electrodes

It involves continuous electrical stimulation of the external anal sphincter via skin surface electrodes or electrodes implanted at the sacral foramina, both of which achieve comparable results. (21) It is a simple and well tolerated modality. The procedure for implantation can be carried out as a day care procedure. (18)

In patients with a diffusely weak sphincter or those who have failed sphincter repair, it has a 70% chance of improvement, if the neuromuscular integrity is preserved. (18) In a large study, 90% of patients had >50% improvement at 2 weeks follow up. This improvement was sustained and 41% were completely continent at 1 year follow up. 83% of 120 patients who went on to follow up had significant symptomatic improvement.(25) At 5 year follow up, 36% of patients have reported complete continence, while upto 89% of patients have reported more than 50% improvement in symptoms. (26)

Surgical Repair

In an acute setting, the management will centre around the decision to perform a defunctioning stoma with debridement of the wound. Pelvic floor repair should be done as an elective procedure 3 months after the initial injury to allow for the acute inflammation to subside. (18) Surgical options for anal sphincter injuries include overlap sphincteroplasty, anterior levatorplasty, graciloplasty. (20)

Upto 73 % of patients who underwent surgical repair of the sphincter had good outcomes at a single centre series from south India. Patients with a structural anal sphincter defect had a good outcome following overlap sphincteroplasty. Addition of a levatoroplasty improved the outome in a subset of patients. However, necessity of a gracillis augmentation and history of a previous failed repair portends a poor outcome.(20) Prior series and meta-analysis which assessed the outcomes of sphincter repair surgery showed poor long term outcomes with failure rates upto 86% at 5 years. (27-29)

Colostomy

This is a last resort in treating patients with refractory symptomatic faecal incontinence. Though it causes complete cessation of incontinence, patients report a poorer quality of life as compared to controls. (21)

Animal models in the study of faecal incontinence

Reliable, reproducible and sustainable animal models are required to evaluate the therapeutic efficacy of novel options.(2) To this end, various animal models have been developed to study faecal incontinence in various animals, most commonly rats and dogs. Various methods have been employed to create the anal sphincter injury – electrocoagulation, cryoinjury, surgical excision.

A study in 12 week old Fischer rats demonstrated that cryoinjury of a 90˚ sector of the sphincter using a 3 mm diameter aluminium rod for 30 seconds, repeated after 24 hours produced sustainable deficiency in the anal sphincter. (11) In a three way head to head comparison study of electrocoagulation, cryoinjury and microsurgical excision, sphincter injury was complete and sustained in the microsurgical excision group. (2) Similar models have also been developed using rabbits and dogs as well. (30-34)

Regenerative Medicine in the treatment of incontinence

Maintenance of continence is a complex mechanism involving structural, physiological and neurological components. Thus, regenerative medicine could potentially target any of the targets in the pathway of continence – (1)

1. Restoring the sphincter itself
2. Restoring the pelvic floor support
3. Restoring sphincter innervations

Stem Cells

Stem cells are undifferentiated cells that can differentiate into any specialized tissue under appropriate conditions and replicate to renew itself. (35) They are characterized by two properties (36) –

1. Self-renewal – It enables stem cells to maintain their numbers
2. Asymmetrical division – Out of the two daughter cells produced from a cell division, only one of them proceeds into the differentiation pathway. The other retains its stem cell ability.

Types of stem cells

Broadly, stem cells are divided into two main types –

1. Embryonic stem cells
2. Adult / Tissue stem cells

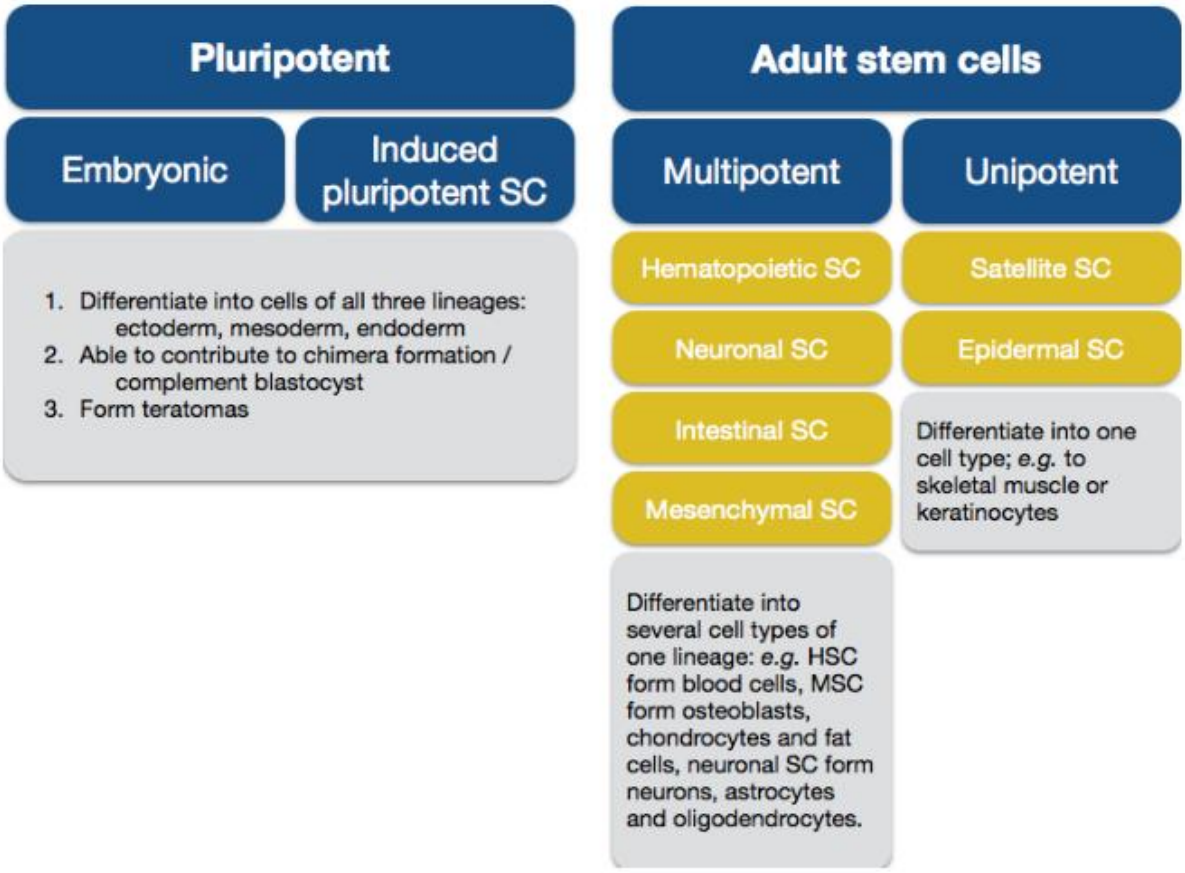


Figure 9. Types of Stem cells
*(Reproduced from Dulak, J., et al. (2015). "Adult stem cells: hopes and hypes of regenerative medicine." ActaBiochim Pol**62**(3): 329-337.)*

Embryonic stem cells are totipotent cells that retain their ability to differentiate into any of the adult tissues. By manipulating the surrounding microcellular environment, they can be made to grow into derivatives from any of the three germ cell layers (i.e. ectoderm, mesoderm and endoderm).

Adult / Tissue stem cells are stem cells residing in specialized niches within adult tissues and serve to replenish the different types of cells within that tissue alone. They

do not have the ability to differentiate into cells of other tissues and hence, cannot be termed pleuripotent cells. (36)

Mesenchymal stem cells are a group of multipotent stem cells that are found in the bone marrow, adipose tissue and other connective tissues. They have the ability to differentiate into any of the stromal cells – chondrocytes (cartilage), osteocytes (bone), adipocytes (fat) or myocytes (muscle). They can be expanded *in vitro* to large numbers and also generate a local immunosuppressive reaction, thus potentially avoiding rejection. Hence, they are commonly used to generate connective tissue for acellular scaffolds in regenerative medicine.(36) Additionally, under specific cellular microenvironments, they have been shown to express markers of cells of other lineages as well, including endothelium, neurons, hepatocytes and kidney epithelial cells. Their role in regeneration of other tissues is being studied.(37) Their role in the cell-based treatment of anal sphincter injury and faecal incontinence has been studied in several animal trials and it has shown promising results. (30, 38-43)

Myogenic stem cells are derived from satellite cells that lie between the basal lamina and sarcolemma of adult muscle fibers. These cells are activated to repair muscle in response to injury or the need for muscle growth. (44)The interest in using autologous myoblasts or muscle derived stem cells is driven by two major factors –

1. They do not induce foreign body reaction as they are native to the recipient.
2. Their use is not fraught with the ethical issues as concerning those with the use of embryonic stem cells.(45)

Role of stem cells in treatment of anal sphincter injuries and faecal incontinence

The idea behind the use of Muscle derived Stem cells for the treatment of anal incontinence originated from studies which showed efficacy in the treatment of urinary incontinence (14-20 from Kang 2008) and other studies which showed an improvement in myocardial contractility after an infarction and in animals models of muscular dystrophy, urethral sphincter insufficiency and post infarction myocardial dysfunction.(46-48)

The first study to use Muscle Derived Stem Cells (MDSC) in the treatment of anal sphincter injury was by Kang et al. Using a 3 armed prospective study, they compared the effect of injection of MDSC and no treatment in Sprague Dawley rats. Their findings showed an improvement in the contractility of the muscles of the sphincter as compared to the controls, though the improvement was not statistically significant. Their study also used histology and immunohistochemistry to demonstrate that the muscle fibres that had regenerated had in fact developed from the implanted cells and not *de novo*.(5)

Human studies evaluating the role of stem cells for faecal incontinence are far and few. A study was conducted by Frudinger et al in 10 women with refractory faecal incontinence secondary to obstetric anal sphincter injury. They injected myoblasts into

the area of the sphincter defect under endoanal ultrasound guidance. They assessed Wexner Incontinence score, quality of life and anal manometry as their outcome variables at 1 year and 5 year follow up intervals. At 1 year follow up, they reported significant decrease in the number of bowel movements per day and Wexner incontinence score. The improvement was sustained even at 5 year follow up. (45, 49)

In addition, several studies have evaluated the therapeutic role of stem cells from various sources for the regeneration of a deficient sphincter. The studies thus far have been summarized in the Table 2.

Table 2. Summary of current studies

Author, Year	Subjects	Injury method	Study design	Intervention Tested	Outcome measures	Results
Kang, 2008(5)	3 week old female Sprague Dawley Rats	Cryoinjury	3 armed cohort study 1.Control group 2.Cryoinjury group 3.MDSC injection group	Autologous myoblasts cultured from the gastrocnemius muscles of the rats	Sphincter contractility measured as force transduction of muscle strips at one week follow up	Muscle contractility showed improvement,but it did not reach statistical significance. Histology showed regenerating muscle fibres in the MDSC group as compared to fibrosis in the injury group
Lorenzi, 2008(38)	24 Wistar Furth rats	Left lateral full thickness internal and external anal sphincterotomy	4 armed cohort study 1.Sham operation 2.Sphincterotomy + repair + saline injections 3.Group 2 + injection of bone marrow derived	Bone marrow derived mesenchymal stem cells	Clinical evaluation, histology, response of sphincter strips to chemical and electrical stimulation	Significant improvement in muscle contractility and regeneration with bone marrow derived mesenchymal stem cells

			mesenchymal cells 4.Group 3 + immunosuppressive therapy			
Aghaee-Afshar, 2009(30)	35 white New Zealand Rabbits	Incision in the external anal sphincter	5 groups 1.Human umbilical cord matrix cells 2.Rabbit bone marrow cells in medium 3.Medium only 4.Saline only 5.Control	Human umbilical cord matrix cells; Rabbit Bone marrow cells	EMG, sphincter contractility	Significant improvement was noted with muscle dominant sphincter with rabbit bone marrow cells. Non-significant improvement with fibrous dominant sphincter in human umbilical cord cells.
Kajbafzadeh, 2010(31)	21 Male New Zealand white rabbits	Longitudinal posterior external sphincterotomy – 9mm long	2 armed cohort study	Autologous Muscle Progenitor Cells (MPCs) from quadriceps myofibre culture	1.Sphincter EMG and Manometry at 14,28 and 60 days (3 animals) & 6 months (3 animals) 2.Histopathology at 60 days and 6 months	Significant improvement in the anal manometric pressures with concurrent regeneration on histology

Frudinger, 2010 (45)	10 Women with 3rd or 4th degree obstetric tears with severe anal incontinence Wexner score >=9; Failed conservative management	Existing injury due to Obstetric Anal sphincter injury	Single arm cohort study	Electrical stimulation followed by implantation of Autologous myoblasts cultured from 1 cm^3 muscle biopsy from the Pectoralis muscle	1.Wexner Incontinence score 2.Rockwood quality of life score 3.Mean Anal canal squeeze pressure 4.Mean canal resting pressure 5.Number of bowel movements per day Measured at pre-implantation, 1 month, 6 months and 12 months post implantation	Significant decrease in the Wexner Incontinence score and Number of bowel movements per day over the course of the study. No significant change in the mean or maximum anal canal pressures at one year follow up. Reduction in the mean resting pressure over the one year. No significant change in the thickness of any anal sphincter component over time.

White, 2010(50)	120 virginal female rats	7 mm incision through the anal sphincter complex	Randomised trial – Two groups of 60 animals each – 60 animals for repair and 60 animals for not to repair; Animals were further divided into two groups: 40-microliter injection at the transection site with either phosphate-buffered solution (control) or myogenic stem cells	Myogenic stem cells – Commercially available H9c2 myoblast cell line	Animals were killed at 7, 21, or 90 days, and the anal sphincter complex dissected and analyzed for contractile function – electrical stimulation	Significant improvement in the contractility in animals treated with myogenic stem cells as compared with control group animals
Pathi, 2012 (39)	204 virginal female rats & 20 more as controls	7 mm incision through the anal sphincter complex	Randomised interventional study; 20 animals as controls; Rest divided into 3 groups – control injection, IV MSCs, local injection of MSCs	Rat bone-derived mesenchymal stromal cells	Ex-vivo muscle contractility, IHC and histology	Direct injection of MSCs into the injured anal sphincter resulted in improved contractile function 21

						days after injury compared with controls. IV MSCs did not have a significant improvement in the sphincter contractility.
Kang, 2013 (32)	10 ten week old female Mongrel dogs	Partial extraction of 25% of posterior sphincter by electrocautery	Two armed cohort study	Porous polycaprolactone beads with autologous myoblasts	Manometry and CMAPs[1] of sphincter just before injury and at 3 months. Inferior rectal nerve EMG Histopathology and IHC at the end of months	CMAPs did not show any significant improvement over controls. Anal pressure were higher than the control group but were not statistically significant. The histology of the animals with myoblast beads showed features suggestive of

[1] Compound Muscle Action Potentials

						foreign body reaction.
Lane, 2013(44)	33 Sprague Dawley rats – Nulliparous female rats at 8-10 weeks of age	Procto-episiotomy – 5mm	2 armed study – one group with injury + injection of PBS(control); second with injury + injection of MSCs	Myogenic stem cells	EMG at baseline, 2 weeks and 4 weeks post injury	Myogenic stem cells accelerated the recovery to normal by upto 2 weeks.
Romanis zyn M, 2013 (19)	20-year old male with faecal incontinence due to an old external anal sphincter rupture in a road accident	Trauma	Single subject case report	Myoblast local injection into the defect	Quality of life, EMG signals in the scar area	Persistent incontinence to flatus; but was continent to stools; Improved quality of life; New EMG signals in scar area.
Salcedo, 2013 (40)	70 female rats	Sphincterotomoy; Pudendal nerve crush injury	Four armed study – Only sphincterotomy, only pudendal crush injury, sham sphincterotomy, sham nerve injury	Mesenchymal stem cell injection – IV/IM	Functional recovery – Anal sphincter pressures, EMG	Signficant improvement in anal sphincter pressures and EMG after sphincterotomy but not after pudendal nerve crush injury

Raghavan, 2014 (51)	Athymic rats	Nil	Single armed cohort feasibility study	Bioengineered internal anal sphincter constructs	Implantation of the construct	The construct adhered to the perirectal tissue and remained healthy. There was neovascularization within the construct.
Salcedo, 2014 (41)	Fifty rats	Partial sphincter excision (25%)	Cohort study – Injury and no injury groups	IM or serial IV injections of Mesenchymal stem cells	Anal pressures	Sustained increase in both resting and peak pressures at 5 weeks
Bisson, 2015 (11)	Fischer rats	Cryoinjury	Three armed study – uninjured controls, cryoinjured + PBS, cryoinjury + myoblasts	Myoblast injection	Sphincter pressures	Significant improvement in the sphincter pressures in animals with myoblasts over PBS rats. Sustained improvement till 6 months follow up.
Fitzwater, 2015 (52)	40 female rats	Transection	Two armed study – PBS injection or stem cell injection	Myogenic stem cell injection	Volume of sphincter, contractile force generation	Stem cells increased the contractile force

						significantly without significant increase in the volume of the sphincter.
Frudinger, 2015 (49)	10 Women with 3rd or 4th degree obstetric tears with severe anal incontinence Wexner score >=9; Failed conservative management	Existing injury due to Obstetric Anal sphincter injury	Single arm cohort study	Electrical stimulation followed by implantation of Autologous myoblasts cultured from 1 cm^3 muscle biopsy from the Pectoralis muscle	1.Wexner Incontinence score 2.Rockwood quality of life score 3.Mean Anal canal squeeze pressure 4.Mean canal resting pressure 5.Number of bowel movements per day Measured at 5 years post implantation	Sustained improvement in the Wexner incontinence score and number of bowel movements per day at 5 year follow up
Montoya, 2015 (53)	80 female rats	Anal sphincter transection	4 armed study – 1.Non-repaired controls 2.Hydrogel matrix scaffold with PBS 3.Hydrogel matrix scaffold	Injection of hydrogel matrix scaffold with myogenic stem cells	Electrical field stimulated contractions of anal sphincter complexes at 4 and 12 weeks	Sustained improvement in contractile responses and striated muscle volume in the group with hydrogel scaffold and

			with myogenic stem cells 4.Hydrogel matrix scaffold with type 1 collagen			myogenic stem cells as compared to all other groups
Oh, 2015 (33)	15 healthy male mongrel dogs (19–22 kg; 10 weeks old)	Surgical resection of ~25% of the posterior external/internal sphincter	Three armed cohort study – one arm with sham surgery as control; other with sphincter injury and no treatment; other with injury and intervention	Polycaprolactone beads with autologous myoblasts	Manometry and CMAPs[2] of pudendal nerve just before injury, 1 month after injury and every month for 3 months Histopathology and IHC at the end of 4 months	The transplanted myoblasts had differentiated and had improvement in the anal sphincter pressures
Romanis zyn, 2015 (54)	10 patients with faecal incontinence	Pre-existing injury	Single armed study	Autologous myoblast injection	Subjective improvement, Manometry, EMG examination	Significant recovery of sphincter function and increased squeeze anal pressure and high pressure zone length of

[2] Compound Muscle Action Potentials

						anal sphincter. Subjective improvement in 60% of patients
Kajbafzadeh, 2016 (34)	16 rabbits	Removal of entire anal sphincter complex	Two armed study – 1.Transplanted decellularised scaffolds 2.Scaffolds with myogenic satellite cells	Myogenic satellite cell injection with external anal sphincter scaffold	EMG, Histology at the end of 2 years	No statistical difference in long term follow up. Short term benefits are in favour of myogenic stem cell injection
Mazzanti, 2016 (55)	32 rats	Sphincterotomy	Four armed study with controls	In-vitro expanded Bone marrow derived mesenchymal stem cells; unexpanded bone marrow derived Mononuclear cells	Histology, in-vitro contractility and functional analysis	Both the groups with cells showed significant improvement of muscle regeneration and increased contractile function.
Sun L,2016 (43)	135 female Sprague-Dawley Rats	Partial sphincter excision	4 armed cohort study 1.No treatment 2.Daily electrical stimulation for 3 days	Local electrical stimulation followed by mesenchymal stem cell delivery	Muscle formation, anal sphincter pressures	Electrical stimulation with single mesenchymal cell injection improved both

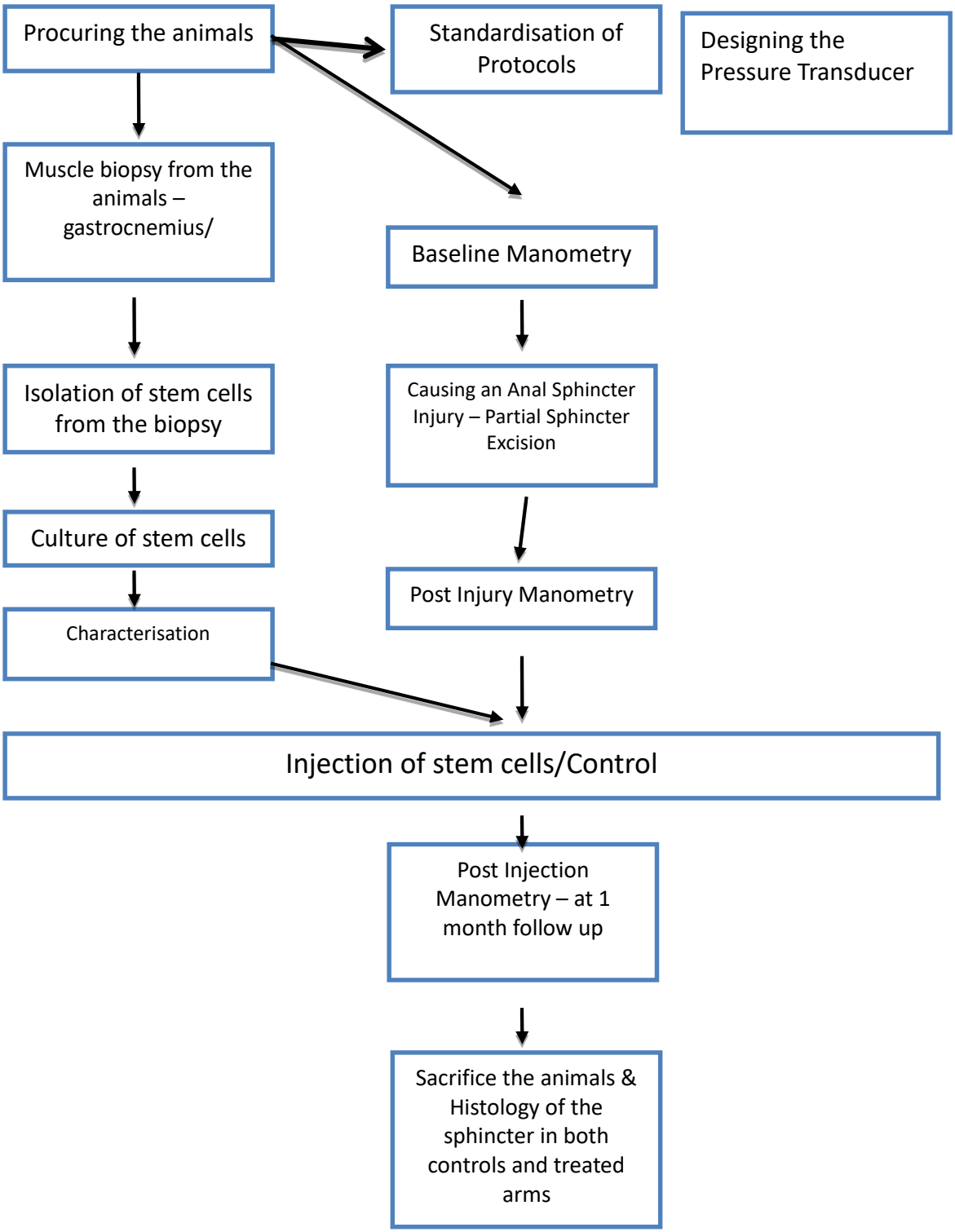
			3.Daily stimulation for 3 days followed by stem cell injection on the third day 4.Daily electrical stimulation followed by stem cell injection on the first and third days.			new muscle formation and anal sphincter pressures at 3 weeks follow up.

Justification for the study

Given the poor long term outcome of conventional treatment with both non-surgical and surgical methods, treatment of anal sphincter injuries and consequent faecal incontinence using autologous muscle derived stem cells presents an attractive treatment option. The studies supporting this line of thought have been summarized above. This intervention, if proven beyond doubt, could offer a potentially safe and effective treatment option for people suffering from a socially disabling condition, thus restoring their productivity, confidence and quality of life.

We hypothesised that injection of autologous myoblast cells into an area of injured anal sphincter would lead to regeneration of the sphincter fibres and improved manometric pressures.

Materials and methods



Study design

The study was a prospective cohort non-randomised study with one control arm with placebo (phosphate buffered saline) and test arm with stem cell injections. All experiments were conducted in Sprague Dawley rats. All surgeries were performed under a laminar air flow chamber to prevent contamination of the sample during harvest for stem cells.

Experimental Procedures

1. Animal Model -

The anal sphincter injury was created by excision of the anal sphincter between 6 and 9 o'clock position. All surgeries were performed with the help of surgical loupes. The step-wise description of the procedure is as follows:

1. The animal was induced with 4-5% isoflurane inhalational anaesthesia and the anaesthesia was maintained with 2-3% isoflurane.
2. A circum-anal incision was made between 6 and 9'clock position 2-3 mm from the anal verge.
3. The mucosal flap was raised creating a plane between the sphincter and the mucosa.
4. The sphincter thus isolated was excised between 6 and 9 o'clock position, as depicted in Figure 8.
5. The wound was left open to heal after achieving haemostasis.

The functional result of the injury was confirmed by pre- and post-injury manometry. Two groups were used – a normal control group that received placebo injections of phosphate buffered saline and a test group which received the MDSC injections. All animals had the muscle harvest and injury under the same anaesthesia.

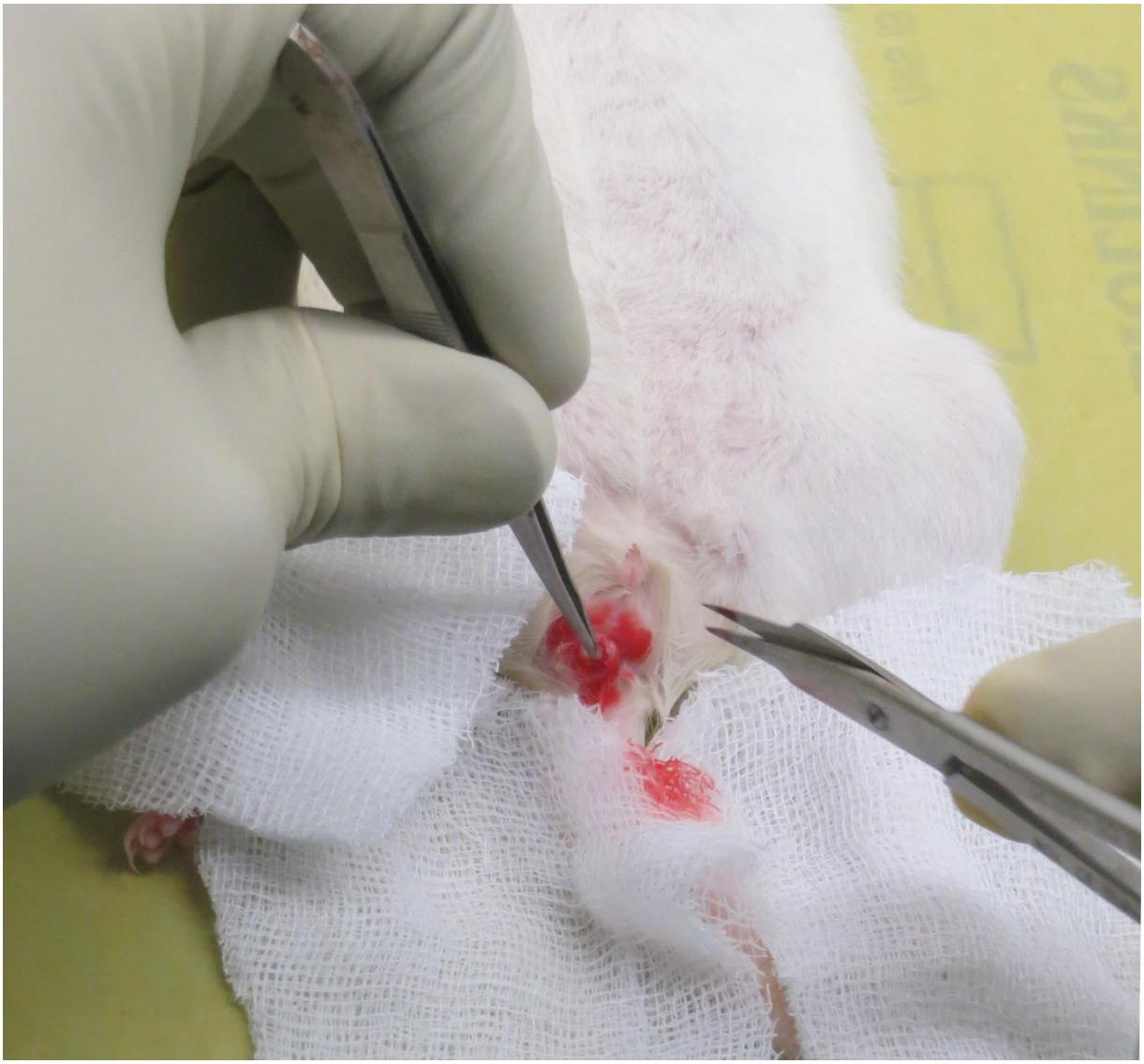


Figure 10. Photo showing partial anal sphincter excision.

Figure 11. Photo showing the left hind limb of animal draped for harvesting the gastrocnemius muscle.

2. Stem Cell Isolation and characterisation-

Stem cells were isolated from the muscles of the hindlimb of the rats (the Gastrocnemius muscles). The hind limb muscles were harvested during the time of the first surgery.

The procedure of the muscle harvest is as follows –

1. The animal is anaesthetized with isoflurane inhalational agent as described above.
2. In supine position, the left hind limb of the animal is prepared with 10% Povidone Iodine and draped.
3. A vertical incision is made on the medial aspect of the left leg for 2 cm parallel to the tibia.
4. The incision is deepened and the gastrocnemius is exposed after dividing the superficial and deep fascia.
5. The muscle is harvested till approximately 1gm of the gross muscle tissue was removed.
6. Haemostasis was achieved. The skin and subcutaneous tissues were approximated in two layers with absorbable sutures (polygalactin).

The isolated bits of muscle were collected into a vial of Dulbecco Modified Eagle Medium and were transported to the lab for further processing.

In laboratory, the muscle sample was transferred to the petri-dish and the contaminating tissues like fascia, vessel were removed using a sterile fine scalpel. The

muscle sample was weighed prior to digestion to calculate the amount of collagenase enzyme required for digestion. According to the muscle weight, required amount of collagenase was weighed (normally for 400mg of muscle, 10mg of collagenase was used). The muscle sample was minced into fine pieces and then transferred to the 50ml centrifuge tube containing collagenase enzyme dissolved in 10ml of muscle culture media (Dulbecco's Modified Eagle's Medium, 10% fetal bovine serum and 1% antibiotic and 1% antifungal). The tube was incubated in $C0_2$ incubator for 2 hours with intermittent shaking. The digested sample was filtered through 100 micron cell strainer to remove the undigested debris and then the filtrate was centrifuged at 2000 rpm for 10 minutes. The supernatant was discarded and the pellet was re-suspended in 5ml of muscle culture media and plated in an Extracellular Matrix (ECM) coated culture flask. The culture flask was maintained in CO_2 incubator with 5% $C0_2$ supplementation.

Cell count

During 80% confluence, cells were washed with 1x phosphate buffered saline and harvested from the flask using 0.25% Trypsin-EDTA. Subsequently, harvested cells was centrifuged at 2000 rpm for 10 minutes and the supernatant was discarded. The cell pellet was re-suspended in 1ml of Dulbecco's Modified Eagle's Medium, from this 20ul of cells suspension was used to count the cells. The cell suspension was mixed with equal volume of trypan blue dye and the loaded on the Neubaur chamber. The chamber was observed under phase contrast microscope and with the aid of the following formula the total number of cells was calculated

$$\left(\frac{\text{Number of cells counted}}{\text{Number of squares counted}}\right) \times \text{dilution} \times 10^4$$

Myogenic differentiation

Functional characteristic of satellite cells were confirmed by myofiber formation. At passage 1, the muscle cells were plated at density of 3000 cells/cm^2 in an ECM coated culture dish and the cells were cultured with muscle culture media. At 80% confluence, muscle culture media was replaced with myogenic differentiation media composed of Dulbecco's Modified Eagle's Medium supplemented with 2% horse serum, 1uM Insulin and 1% antibiotic and 1% antifungal. After four days of culture, cells were observed under phase contrast microscope.

Satellite cells transplantation

The satellite cells were cultured till passage 1 and transplanted into the rat sphincter defect. Primary cells isolated from the muscle were cultured at 5000 cells/cm2 in ECM coated cultured flask contains muscle culture media. At 70-80% confluent, cells were harvested using 0.25% Trypsin-EDTA and re-suspended at concentration of $1x10^6$ cells/ 0.05ml in phosphate buffered saline (1x). The cells were injected into animal defect using BD Insulin Syringe.

3. Treatment of sphincter injury using autologous MDSC Transplantation

The animal was anaesthetized using Isoflurane inhalational anaesthesia. The wounds at the injury site and the harvest site were inspected for signs of healing. The cells isolated as above or Phosphate buffered saline were injected into the centre of the defect, depending on the group assigned to the animal. Following the injection, the animal was brought out of anaesthesia and was returned back to its cage.

4. Functional evaluation of sphincter using manometry

Anal manometry was performed prior to the injury for a baseline reading, immediately after the injury and just prior to the sacrifice of the animal at follow up. All measurements were performed using general anaesthesia without muscle relaxation in supine position. The manometry was performed using a size 4 latex balloon (Kent Industries, USA) connected to a pressure transducer via an arterial line extension. The signals from the transducer were acquired in a digital format by using a data acquisition system (PowerLab 15T, ADInstruments, Spechbach, Germany) and a software Lab Author version 4.5 (ADInstruments, Spechbach, Germany). This setup is shown in Figures 12-14.

Figure 13. Photograph of the ADInstruments Powerlab 15T

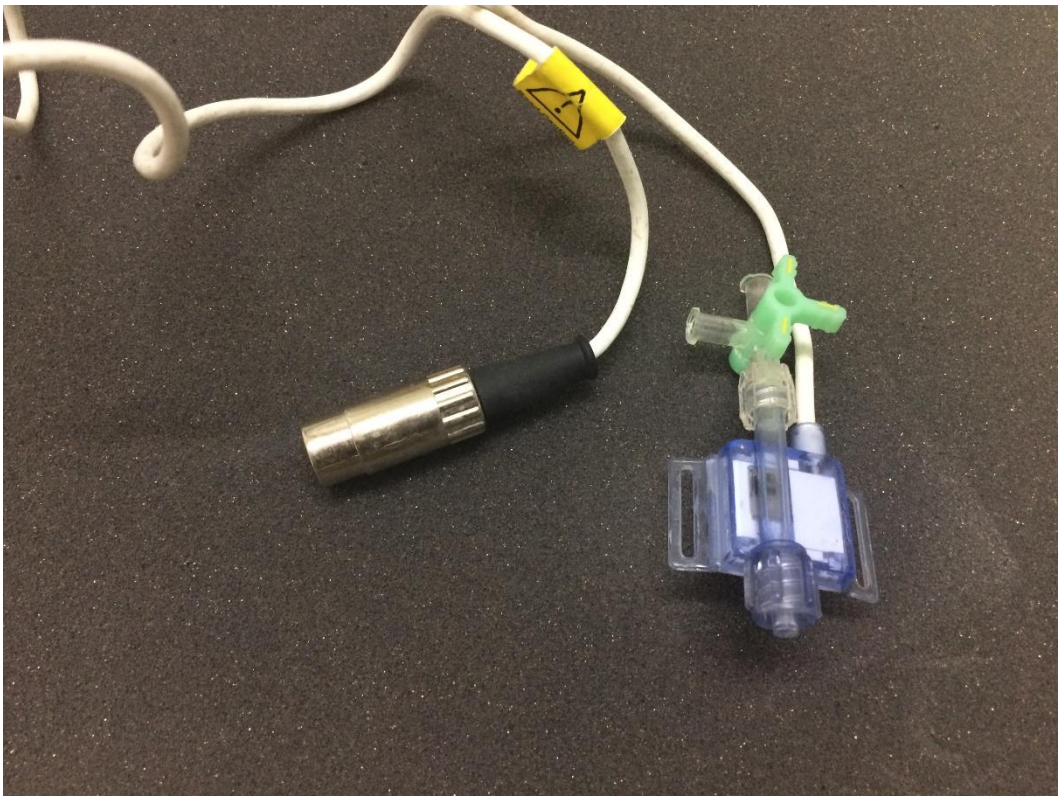


Figure 12. Photograph of the pressure transducer

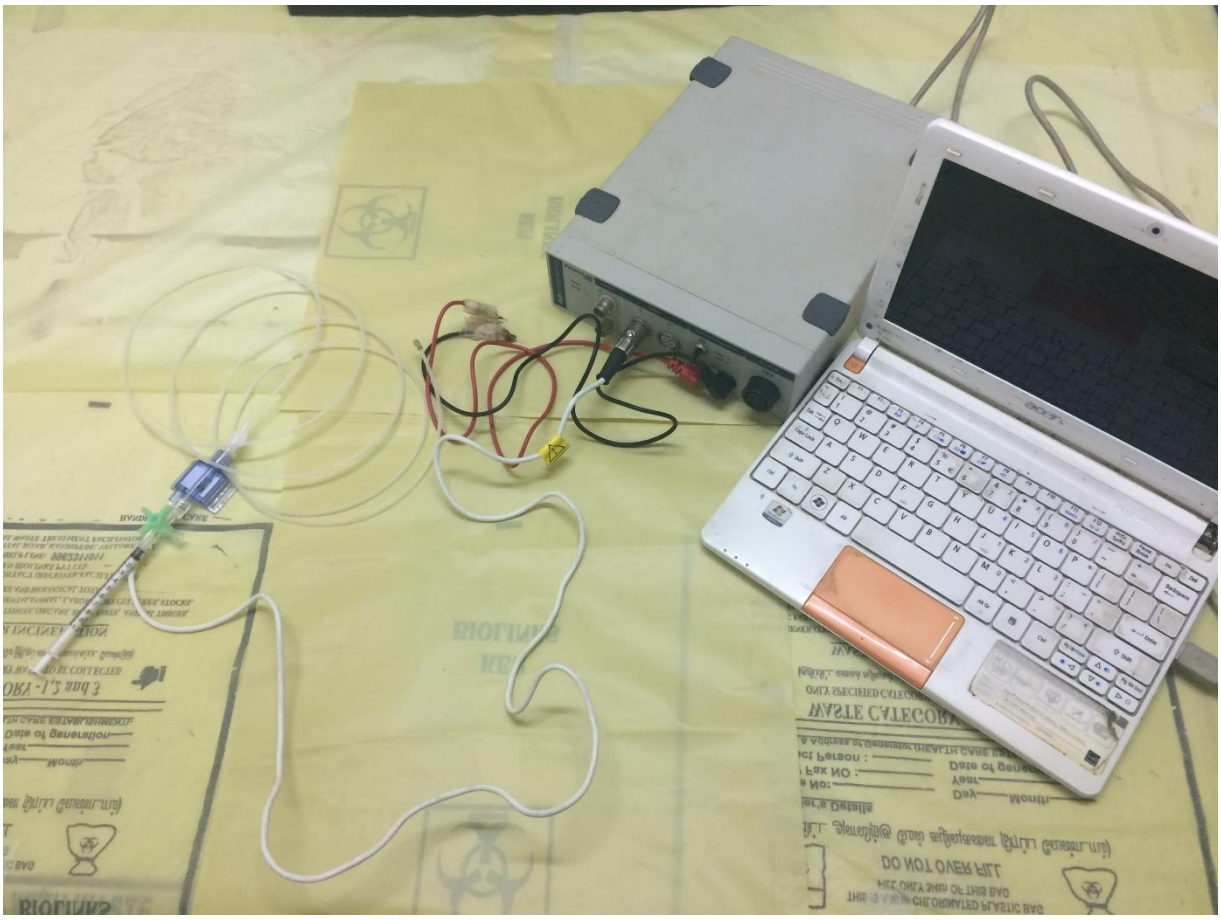


Figure 14. The setup of the pressure transducer with the computer interface

The manometric evaluation was done to evaluate a baseline resting tone as well as response of the muscle to electrical stimulation.

The baseline resting tone was calculated as the resistance offered by the muscle to the stretch offered by an inflated balloon being pulled out. The procedure is as follows –

1. The deflated balloon was inserted into the rectum of the animal past the anal sphincter.
2. The balloon was inflated with 0.1 ml of water.
3. The balloon was then pulled out of the rectum across the sphincter in a smooth manner.
4. The incremental response in the voltage generated on the graph was taken as representative of the baseline resting tone of the anal sphincter.

The response to electrical stimulation was measured as the increment noted in the graph when the muscle was stimulated to contract around the balloon. The procedure is as follows:

1. The latex balloon was positioned such that the widest part of the balloon was in the anal canal.
2. Two electrodes were used – one electrode in the muscles of the right thigh and the second electrode placed at 6 o'clock position into the anal sphincter 0.1 cm from the anal verge.

3. The sphincter was stimulated using 20 mA of current using the isolated stimulator mode of the LabAuthor software and PowerLab 15T hardware with frequency of stimulation at 1 Hz for 5 pulses.
4. The incremental increase in the voltage signal generated was noted as the electrical stimulation pressure, expressed in millivolts (mV).

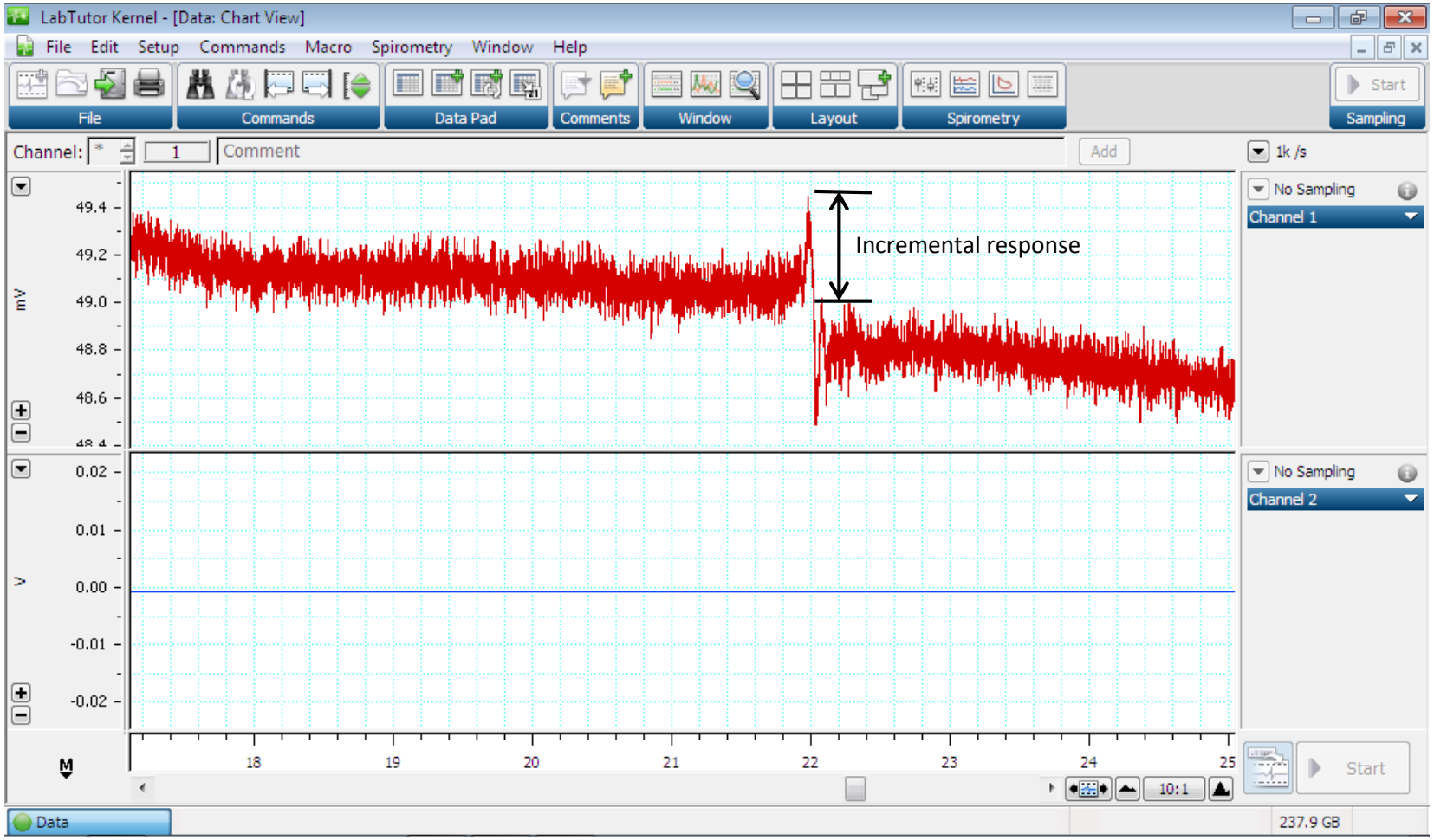


Figure 15. Screenshot showing incremental response during measurement of baseline anal sphincter tone.

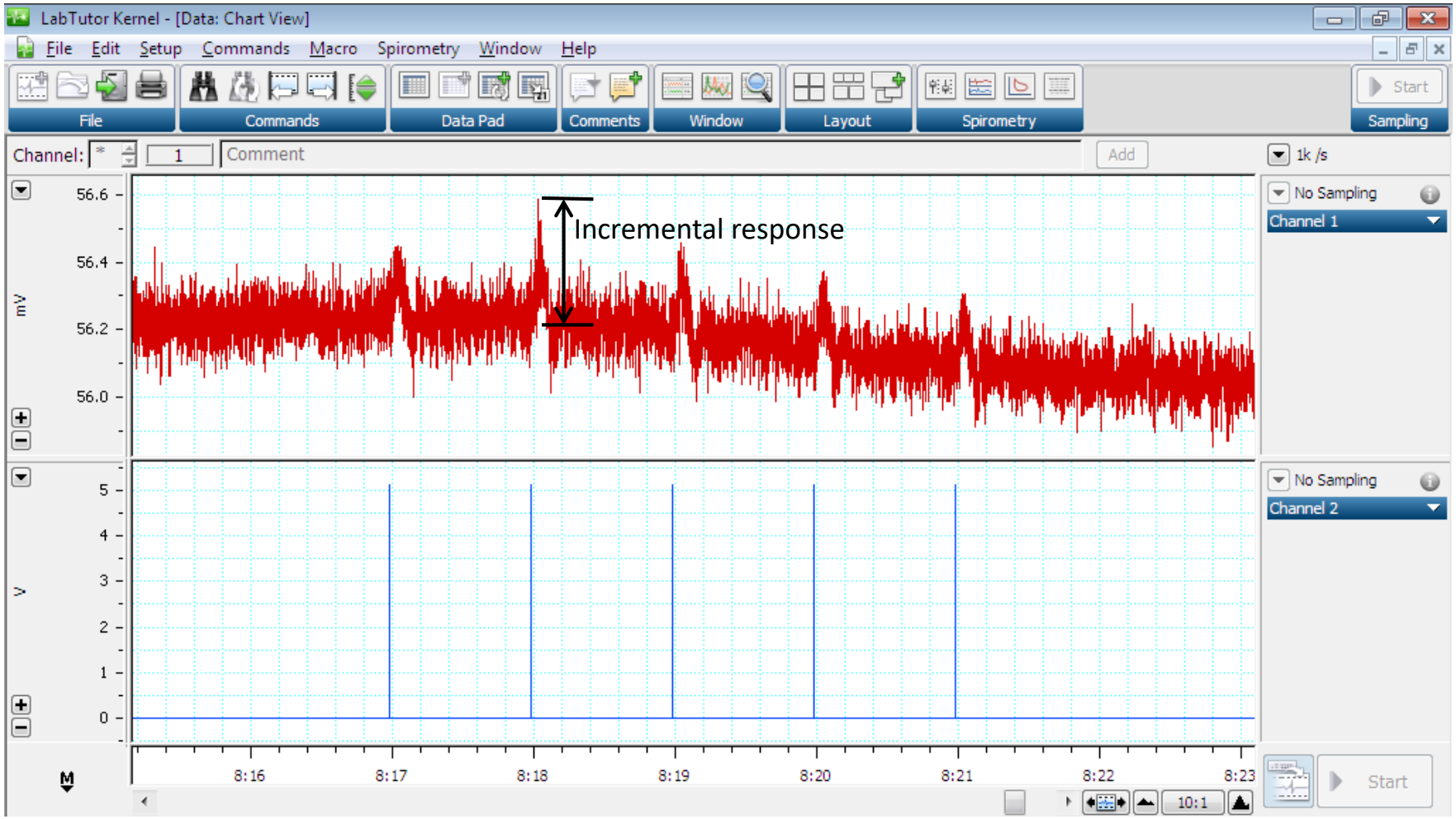


Figure 16. Screenshot showing incremental response during measurement of anal sphincter tone following electrical stimulation.

5. Histopathological evaluation

The animals were sacrificed at the end of the follow up using carbon dioxide overdose. The lower rectum, anal canal along with the anal sphincter complex was surgically removed through a circumanal incision for histopathological examination. The specimen was then fixed in 10% buffered formalin. Prior to embedding into paraffin, the specimen was divided into two or three parts based on the length and suitability for making the histopathology paraffin blocks. Each section was stained using two different coloured inks – Black at 12 o'clock position and green at 3 o'clock position to aid in the orientation and identification of the site of the defect.

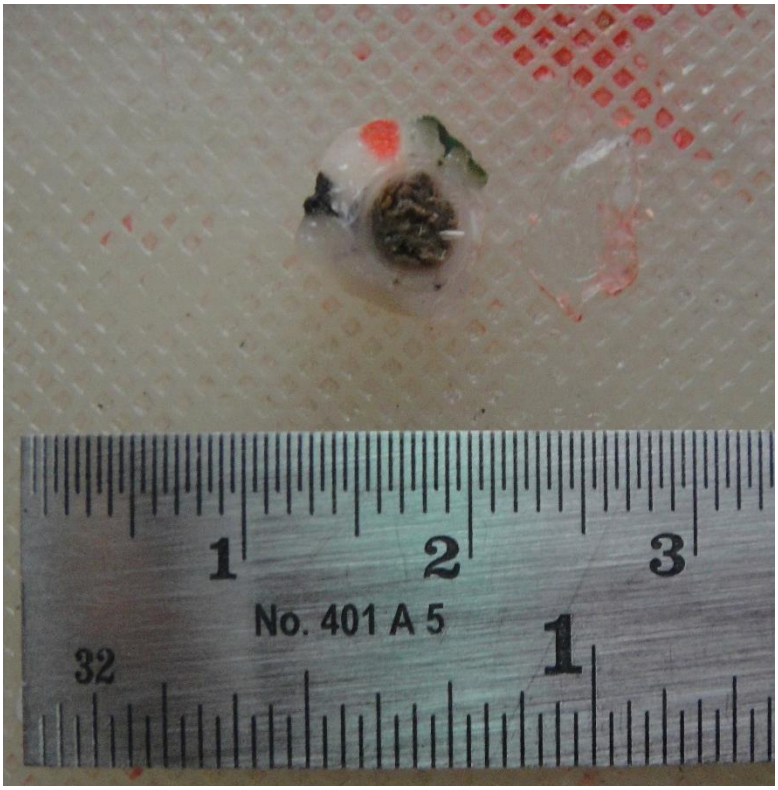


Figure 17. Specimen from the anal canal complex with the orientation inks on it.

The blocks were sectioned into 5 µm sections using a microtome (Leica Biosystems, Nussloch, Germany). The sections were mounted onto slides and stained with haematoxylin and eosin. The specimen was oriented in an end on fashion such that the entire circle of the anal canal with the surrounding musculature could be seen in a single section.

The slides were evaluated for presence of the sphincter defect, presence of fibrosis, signs of foreign body giant cell reaction, signs of regeneration and presence of muscle fibres in the area of the defect.

6. Statistical analysis

Analysis was conducted using descriptive statistics using Microsoft Excel (Microsoft Inc, Redmond, Virginia, USA). The manometry pressures are expressed as median ± standard deviation and the individual rat's data has been represented in a graph created using Microsoft Excel. The histopathological data is primarily presented in a narrative fashion in view of small numbers and lack of definitive objective criteria to pool in results.

Results

A total of 11 rats were used for the study – 6 in the control arm and 5 in the treatment arm. The details of the rats with respect to gender, age and weight at the time of injury are given in Tables 3 and 4. The average age of the animal at the time of the surgery was 11.36 weeks and the average weight was 276.4 gm. There were 2 males and 9 females in the total number of animals.

Table 3. Details of Control Rats

Rat No.	Gender	Age at Surgery	Weight at Surgery
C1	M	14 weeks	500 g
C2	F	14 weeks	275 g
C3	F	14 weeks	200 g
C4	F	11 weeks	260 g
C5	F	11 weeks	280 g
C6	M	11 weeks	360 g

Table 4. Details of Test Rats

Rat No.	Gender	Age at Surgery	Weight at Surgery
T1	F	6 weeks	185 g
T2	F	11 weeks	250 g
T3	F	11 weeks	225 g
T4	F	11 weeks	250 g
T5	F	11 weeks	255 g

Culture of Stem Cells

Gastrocnemius muscle was harvested from 5 animals and the satellite cells were isolated using a protocol established in our laboratory. A mean weight of 408 mg of gastrocnemius muscle was harvested from five rats and a mean of 2.31 x 10^6 cells were isolated from these muscle samples (Table 5).

Table 5. Muscle sample weights and cell counts from the cell culture animal group

Rat	Muscle (weight in mg)	Cell Count
T1	300	2585000
T2	470	1690000
T3	420	2275000
T4	450	975000
T5	400	4070000
Mean	408	2,319,000

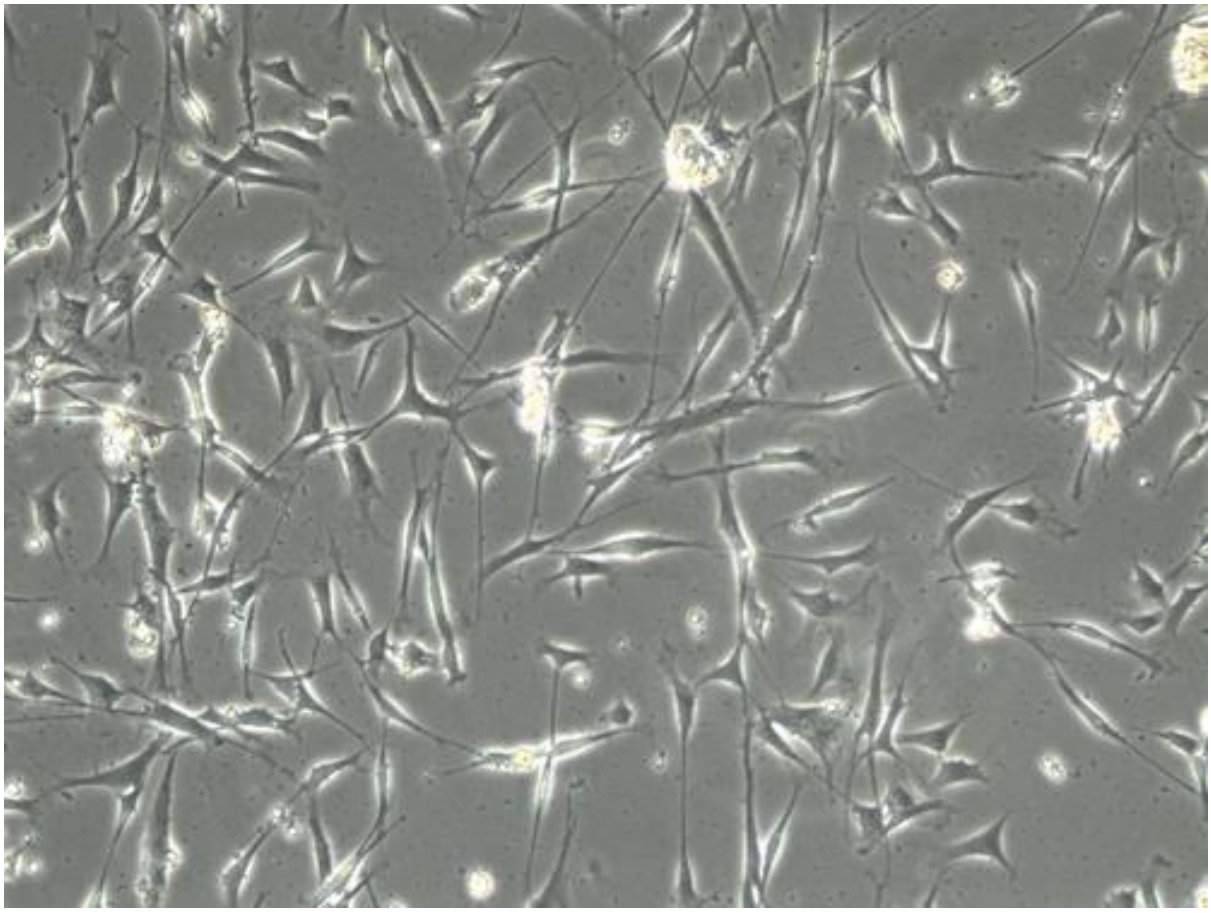


Figure 18. Phase contrast microscope image of spindle shaped satellite cells on day 3, isolated from the rat gastrocnemius muscle. Passage 1. Magnification 10x

Myogenic differentiation

During myogenic differentiation, spindle shaped satellite cells were fused with neighboring cells and form multinucleated myofiber, this property confirms functionally that the isolated cells are satellite cells. In this study, the cells isolated from gastrocnemius were cultured at high density in myogenic differentiation media and on day 5, myofibers formation were observed. This confirmed that the isolated cells are satellite cells.

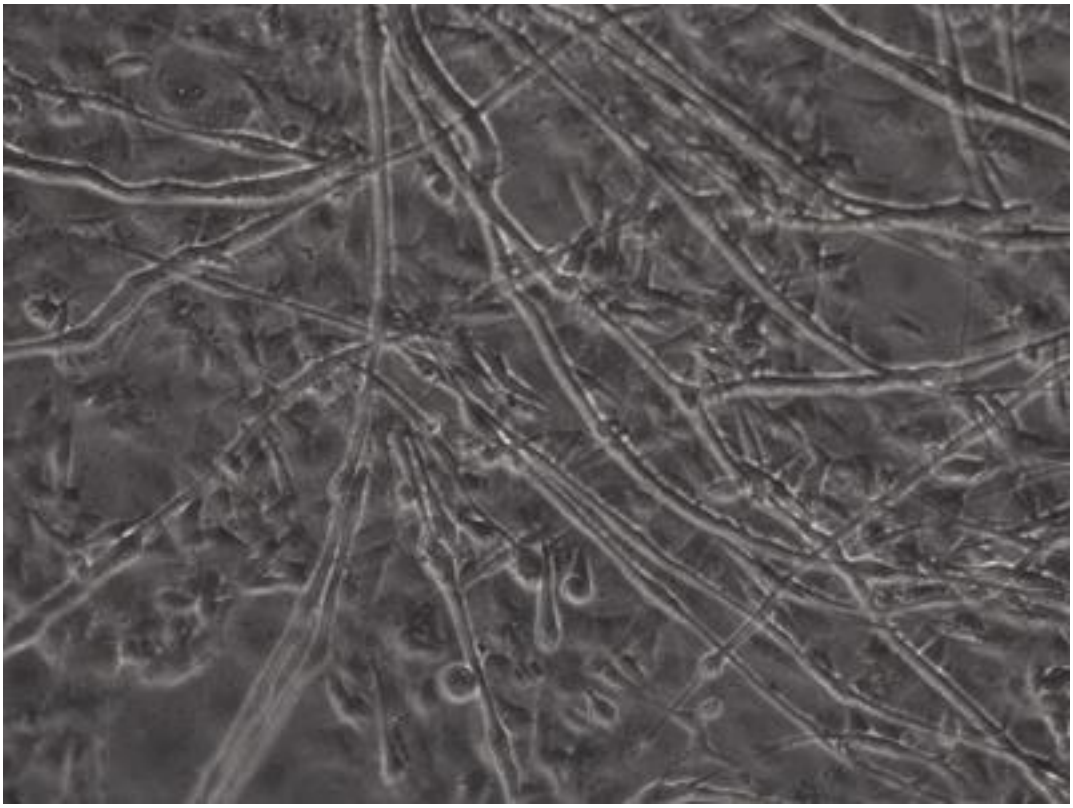


Figure 19. Phase contrast microscope image of mature myofibers formed during myogenic differentiation of satellite cells. Day 4, magnification 10x.

Functional Outcomes Using Manometry

The details of the manometry are given in Tables 3 and 4. The efficacy of the partial anal sphincter excision was evaluated by comparing the preoperative manometry data with the data obtained in the immediate postoperative period.

In control animals, it was demonstrated that the median value of anal sphincter basal pressures decreased from 0.48 mV to 0.14 mV after the injury. This improved to normal values (0.49 mV median value) at the end of a median follow up period of 4 weeks.

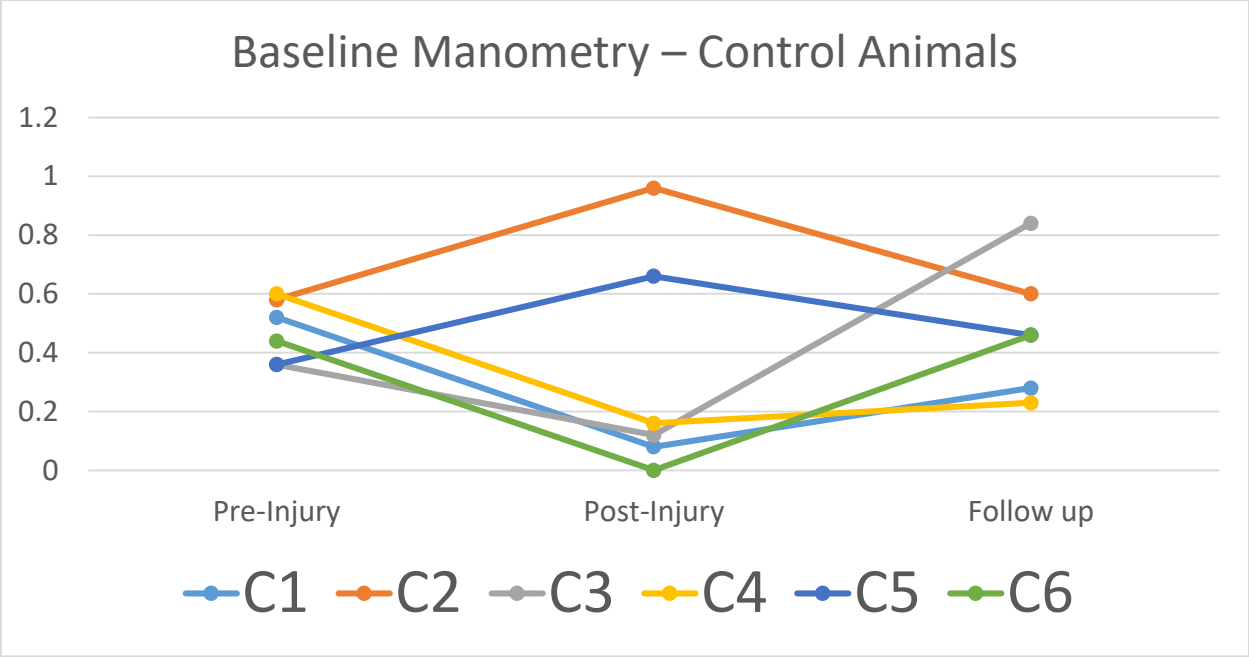
In test group of animals, it was demonstrated that the median value of anal sphincter basal pressures decreased from 0.48 mV to 0.14 mV after the injury. This improved to normal values (0.49 mV median value) at the end of a median follow up period of 4 weeks.

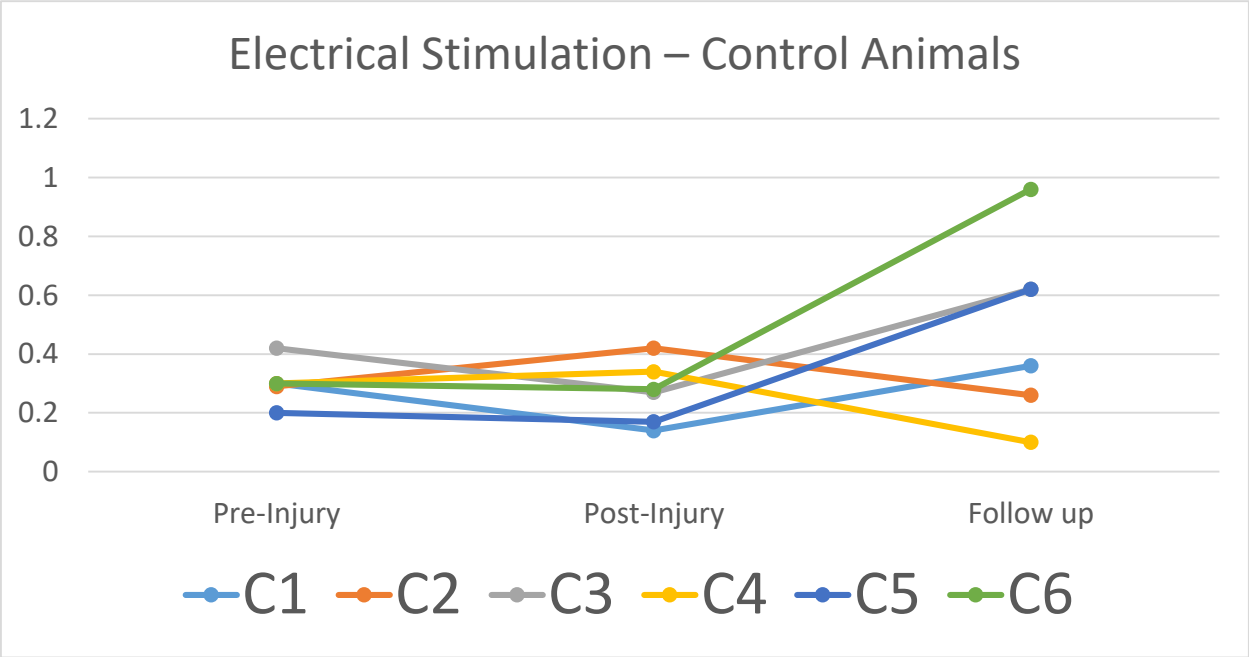
Table 6 - Anal manometry of Control Group

Rat No	Pre-injury		Post Injury		Time of follow up (in weeks)	Follow up	
	Baseline (mV)	Electrical Stimulation (mV)	Baseline (mV)	Electrical Stimulation (mV)		Baseline (mV)	Electrical Stimulation (mV)
C1	0.52	0.3	0.08	0.14	3	0.28	0.36
C2	0.58	0.29	0.96	0.42	3	0.6	0.26
C3	0.36	0.42	0.12	0.27	3	0.84	0.62
C4	0.6	0.3	0.16	0.34	6	0.23	0.1
C5	0.36	0.2	0.66	0.17	6	0.46	0.62
C6	0.44	0.3	0	0.28	5	0.46	0.96

Table 7- Anal manometry of Test Group

Rat No	Pre-injury		Post Injury		Time of follow up	Follow up	
	Baseline (mV)	Electrical Stimulation (mV)	Baseline (mV)	Electrical Stimulation (mV)		Baseline (mV)	Electrical Stimulation (mV)
T1	0.176	0.2	0.95	0.16	4	0.34	0.3
T2	0.31	0.11	0.19	0.09	6	0.43	0.49
T3	0.2	0.38	0.06	0.15	6	0.3	0.7
T4	0.5	1.08	0.08	0.21	6	0.26	0.47
T5	0.52	0.3	0.23	0.27	6	0.22	0.12


Baseline Manometry – Control Animals
1.2
1
0.8
0.6
0.4
0.2
0
Pre-Injury
Post-Injury
Follow up
C1
C2
C3
C4
C5
C6


Electrical Stimulation – Control Animals
1.2
1
0.8
0.6
0.4
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0
Pre-Injury
Post-Injury
Follow up
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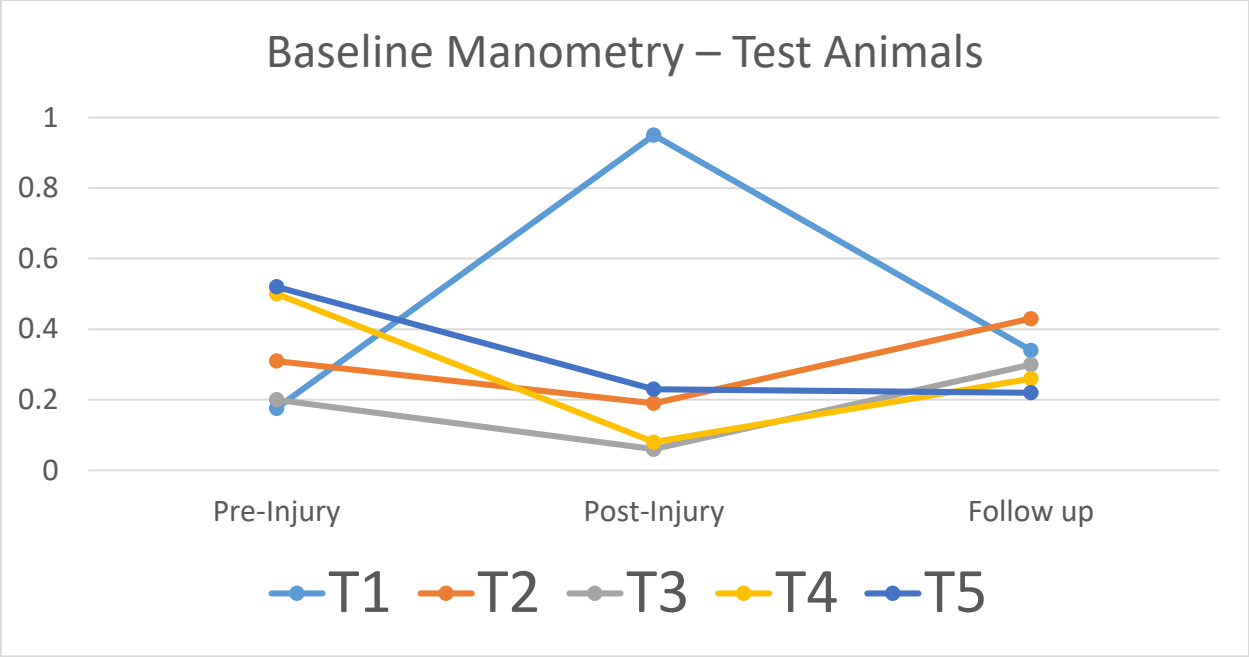
C2
C3
C4
C5
C6


Baseline Manometry – Test Animals
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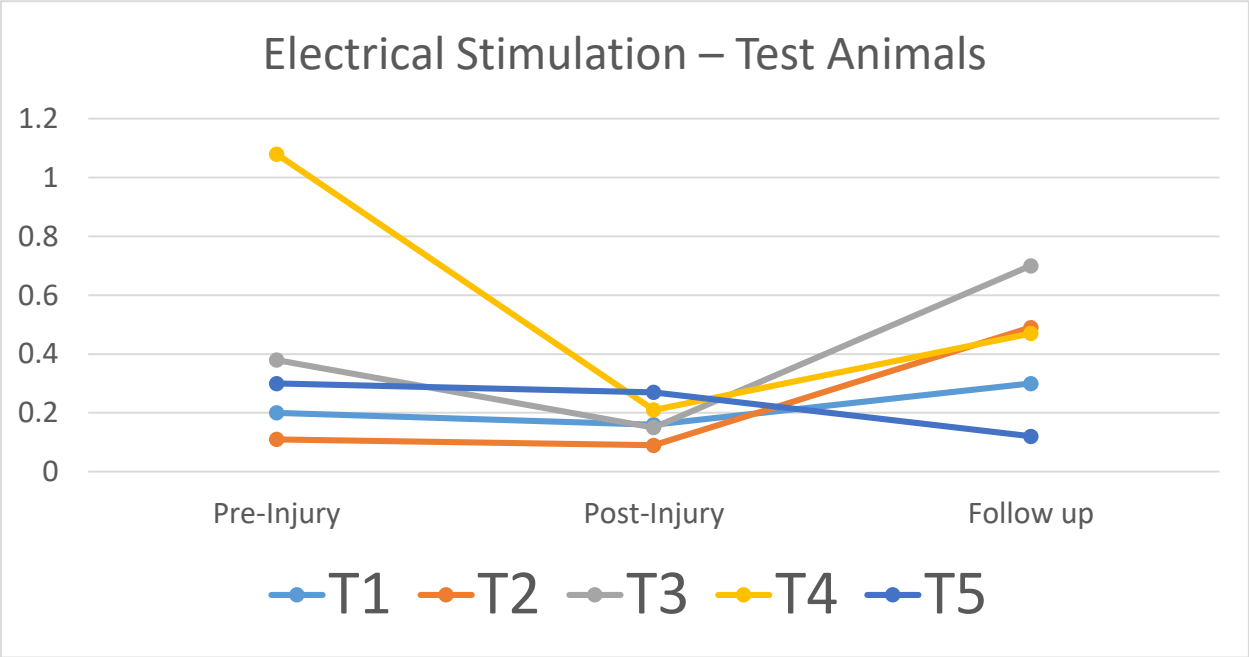
Electrical Stimulation – Test Animals
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Pre-Injury
Post-Injury
Follow up
T1
T2
T3
T4
T5

Histological findings using histopathology and immunohistochemistry

The histopathological evaluation of the specimens was carried out on slides stained with both Haematoxylin and Eosin as well as Masson-Trichrome staining techniques. They were then independently assessed by a pathologist who was blinded to the assignment of the animal using 4 guiding parameters - infiltration of inflammatory cells, cell proliferation, structural regeneration and fibrosis.

Histopathological examination of some of the the control animals shows a clear area of defect with mild to moderate fibrosis in the area of defect. This has been shown in Figures 17 & 18.

In the test group of animals, a clear area of defect with unorganized muscle interspersed between the fibrotic tissues is noted concurrently with inflammation and giant cell reaction. This has been shown in Figures 19 & 20.

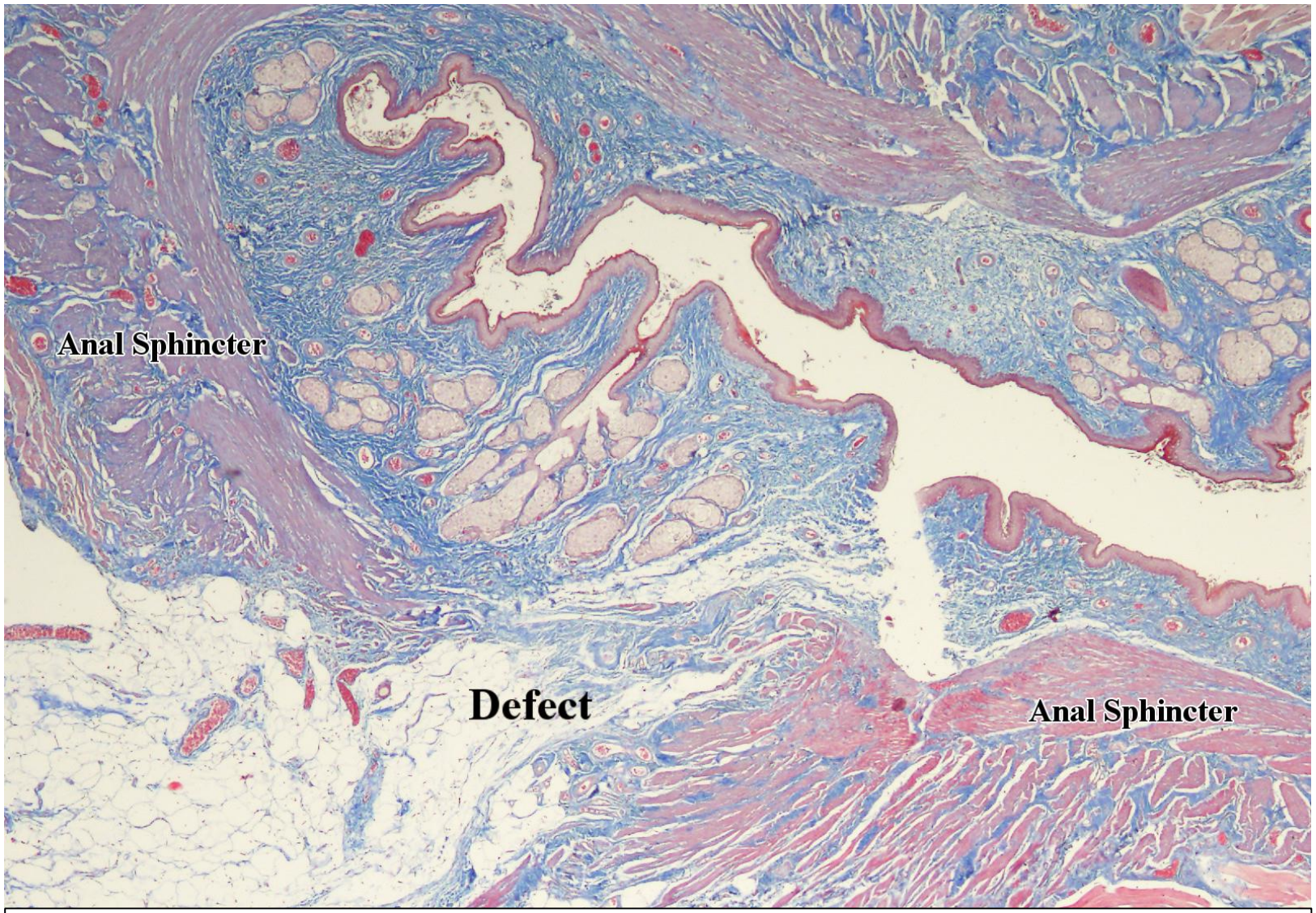

Figure 20. Cross section of anal sphincter in a control animal at one month. Masson-Trichrome stain; Low power magnification (4x)

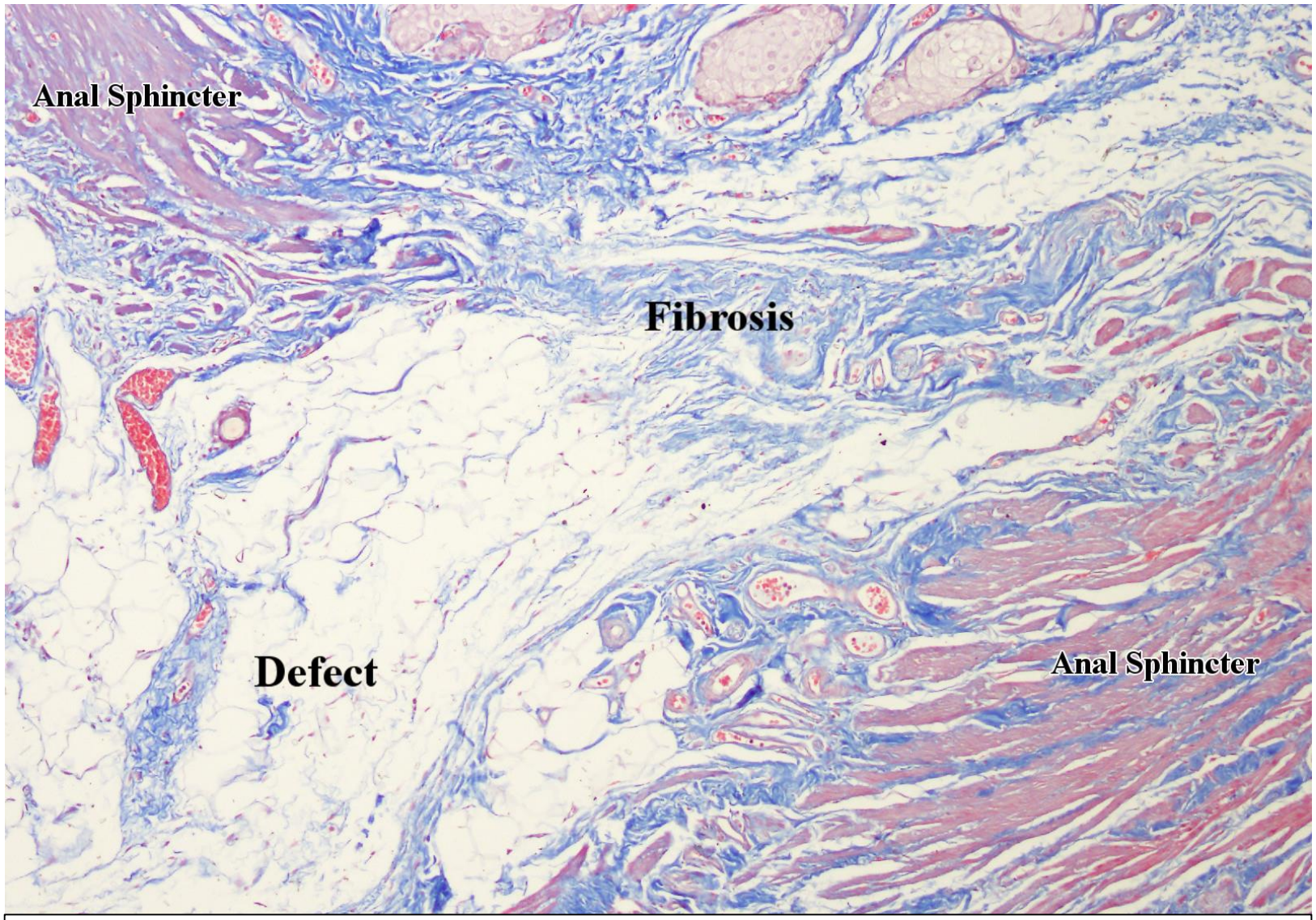

Figure 21.Cross section of anal sphincter in a control animal at one month. Masson-Trichrome stain; 10x magnification

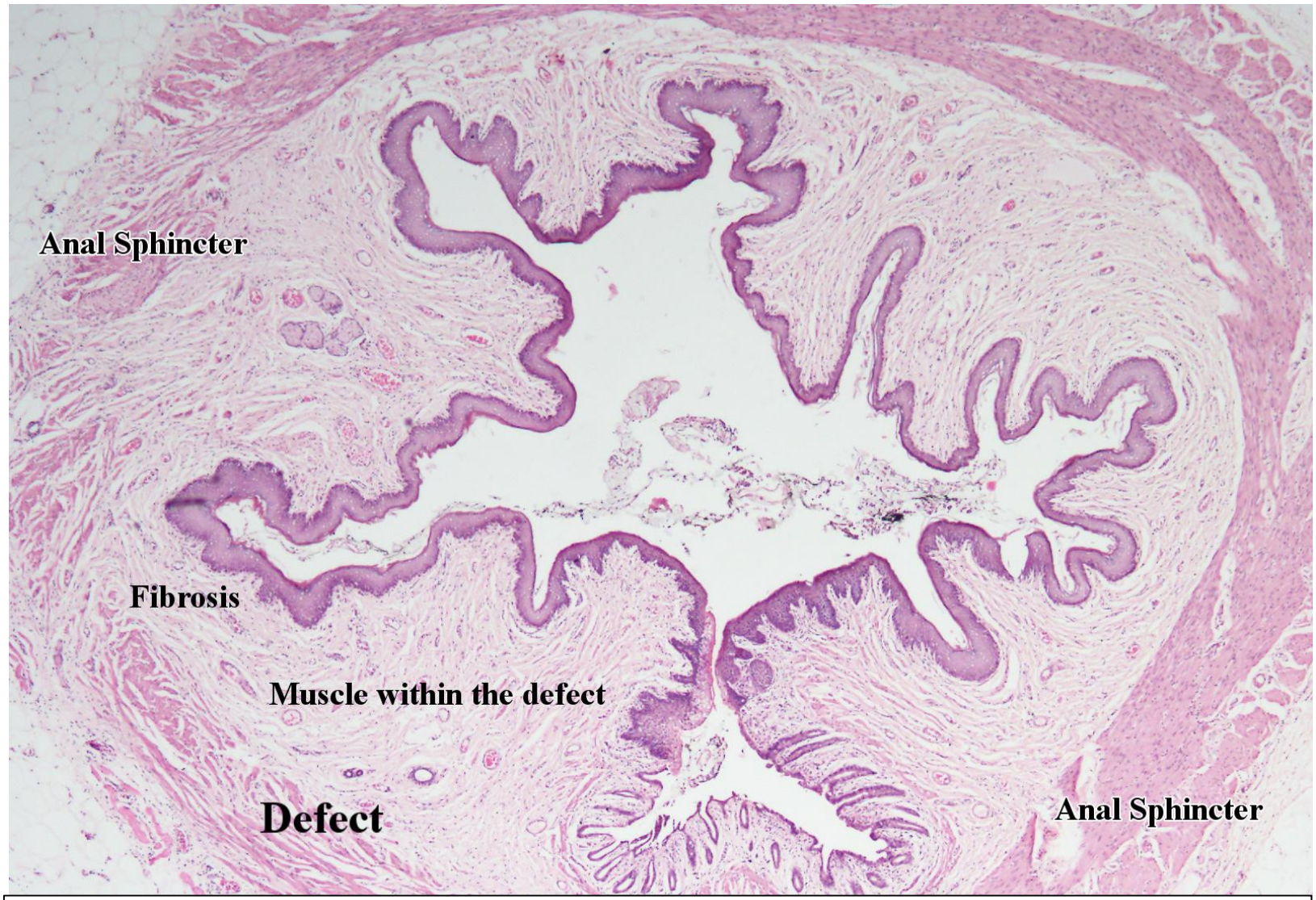

Figure 22.Cross section of anal sphincter in a test animal at one month. Haematoxylin-Eosin stain; 4x magnification

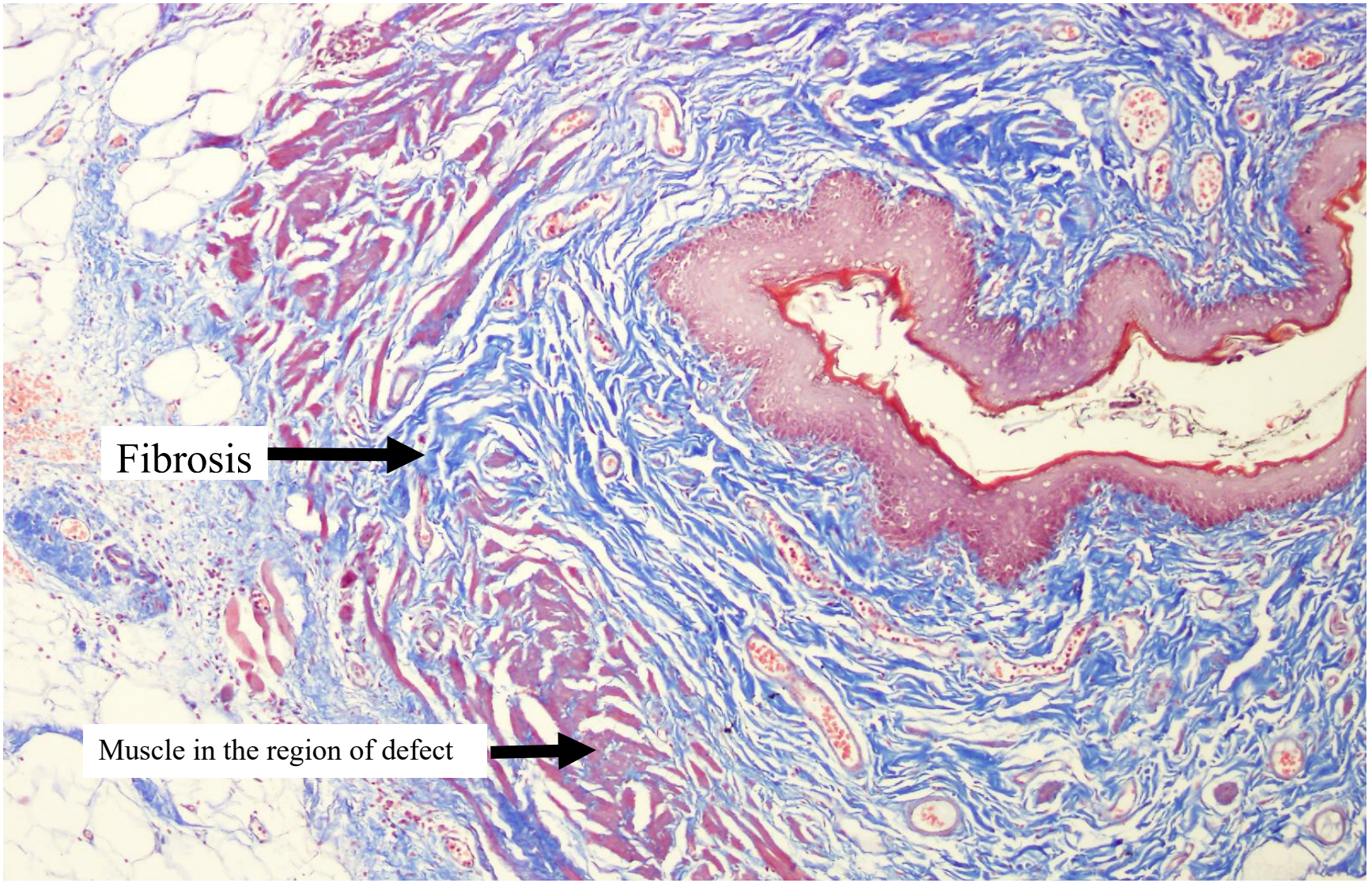

Figure 23.Cross section of anal sphincter in a test animal at one month. Masson-Trichrome stain; 10x magnification

Discussion

Treatment of faecal incontinence using stem cells is a new addition into the armamentarium of the various available treatment options. However, it needs prior validation among animal studies. This study serves as an effective pilot study for further research involving rat anal sphincter injury and regeneration using stem cells. The injury method of partial sphincter excision has been attempted for the first time in India. While it has shown accurate results, the reliability is yet to be demonstrated. Decrease in the baseline and electrical stimulation sphincter pressures following injury in most of the animals is proof that this injury method results in significant anal sphincter injury. However, the increase following the injury in certain animals could possibly be secondary to inadequate injury to the sphincter or an error in the measurement. There is a definite learning curve involved in understanding the anatomy of the rat anal sphincter.

Isolation of stem cells from the rat gastrocnemius muscle has been consistent among all the samples and the cell culture data supports this statement.

Anal manometry showed a decrease in the sphincter pressures in both baseline and electrical stimulation in both control and test arms after the injury. At a median follow up period of 4 weeks, anal manometry shows that both the control and test animals recover in their baseline and electrical stimulation pressures to pre-injury values. Such an improvement in the control group could be explained by a couple of mechanisms.

The injected cells or the phosphate-buffered saline could potentially act as a bulking agent and increase the sphincter pressures artificially without an actual increase in the bulk of the sphincter. There could also be *de novo* regeneration of the sphincter from satellite cells from the native sphincter.

In the test group of animals, such a recovery could potentially be interpretated as proof of sphincter regeneration. However, the cells injected could potentially increase the activity of the local stem cells via paracrine secretions and thus lead to muscle regeneration. This would mean that the cells injected are not directly regenerating into new cells, rather it is the satellite cells that have pre-existed in the native sphincter complex that have regenerated back. This differentiation would require the use of a live cell marker and *in vivo* tracking of the cells.

Limitations of the Study

This study was performed only with 6 control animals and 5 test animals. Due to the small sample size and inherent inter-animal variability in rats, the reliability of these findings is in question. The study also tests a novel surgical procedure, not performed prior to this study, from the indian subcontinent. The novelty of protocols necessitates that they be standardized in the beginning and this study has done just that.

To definitively prove that regeneration of muscle from injected stem cells and not *de novo* stem cells from the anal sphincter complex, it would be mandatory that the cells injected be labelled with either a fluorescent or radiomarker to enable *in vivo* tracking of these cells. However, in view of logistical constraints, that could not be performed in this study.

Conclusion

Muscle derived stem cells (MDSC) can be potentially used as a therapeutic modality for treatment of anal sphincter injuries. Our study raised the curtain on a few findings –

1. A rat model of anal sphincter injury is a viable model wherein the functional and histological parameters can be monitored to study the therapeutic effect of an intervention.
2. Partial sphincter excision of one quarter of the sphincter led to a decrease in sphincter pressures in most animals. However, this was not corroborated by manometric pressures in a few animals where a paradoxical increase was noted. This may have been due to error in the measurement or inadequate sphincter injury.
3. Gastrocnemius muscle is an easily accessible and reliable source to isolate autologous muscle derived stem cells (MDSCs).
4. The anal sphincter pressures returned to normal values in both the control and test group of animals by the end of the follow up period.
5. Injection of autologous muscle derived stem cells(MDSCs) showed evidence suggestive of muscle regeneration in the area of defect. However, this requires further research to confirm the source of the regenerated muscle.

While the data is still preliminary, the options for treatment of anal sphincter injuries continue to improve and be innovative. Further basic science and translational research is needed to confirm and validate our findings in large animals prior to human clinical trials.

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